In the name of Allah the Merciful and Beneficent

An Investigation of the Effect of Short Bouts of Exercise on Adiponectin Concentrations in Young Healthy Females

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Abstract

White adipose tissue is not just a storage organ. It is now recognised as an endocrine organ. It secretes many substances known as adipokines, which are thought to link obesity with type 2 diabetes (T2D). One of the most important adipokines is adiponectin. It is a peptide hormone consisting of 244 amino acids with molecular weight of 30 KD. It circulates in plasma in high concentrations (3-30 µg/ml). Adiponectin polymerises to form many bigger forms. Those are low molecular weight (LMW); middle molecular weight (MMW) and high molecular weight (HMW). The HMW adiponectin is the active form of the hormone.

The concentrations of most adipokines are increased in obese people. Adiponectin is unusual in that its concentration is lower in obese people. Consequently its concentration is decreased in some related metabolic disorders. Its concentrations decrease in cardiovascular diseases, diabetes, dyslipidemia and insulin resistances. It is well known that exercise increases insulin sensitivity, also adiponectin was reported to regulate insulin. The effect of exercise on the adiponectin concentrations in plasma is controversial, but the extent to which the exercise regulates the interstitial adiponectin concentrations is not fully examined. The main site of adipokines secretion is adipose tissue. Therefore the study of these substances at the site of their production has a special interest. Recently, microdialysis techniques have been extended to become important in the measurement of substances in the extracellular fluid of many tissues such as subcutaneous adipose tissue. In particular, it has been used for measurement of adipokines.

This thesis includes three studies. The first study was aimed at examining the effect of one hour of moderate exercise at 50% of maximum oxygen consumption v on adiponectin concentration in dialysate samples taken from subcutaneous abdominal adipose tissues (SCAAT). 15 healthy young female volunteers, age 22.8 ± 3.0 years (mean ± SD) participated in this experiment divided into two groups depending on their body mass index (BMI), a lean group BMI 22.2 ± 1.6 kg/m^2 (mean ± SD) and an overweight group BMI 27.7 ± 1.9 kg/m^2 (mean ± SD). The samples were collected using CMA 66

microdialysis catheters with membrane cut off 100 KD. Fitness assessment was done for all volunteers about one week before the main trials. The main trials were done on two consecutive days, a rest day and an exercise day. Each day lasted for 4 to 6 hours. On the first day the microdialysis catheter were inserted in abdominal subcutaneous tissue 4 cm lateral to the umbilicus on the left side. Dialysate samples were collected every 30 - 45 minutes. On the exercise day volunteers exercised for one hour at 50% $\dot{V}O_{2\,max}$. All samples were analysed for adiponectin concentrations using Mercodia ELISA technique.

The principle findings of this study were that CMA 66 microdialysis catheters worked effectively for two consecutive days for fluid recovery. Adiponectin concentrations were very low and varied, in same volunteer from time to time, and between volunteers. However, the statistical analysis showed no significant difference in adiponectin concentrations between lean and overweight groups. Adiponectin concentrations in the first two samples on the first day of the insertion were significantly higher than the first two samples on the second day of the insertion. Finally, adiponectin concentrations in dialysate samples recovered by 100 KD microdialysis catheters were very low. Therefore, the effect of the exercise was not clear.

The second study aimed to compare the adiponectin concentrations in plasma and dialysate samples. Six healthy male volunteers age 32.8 ± 13.1 years and BMI 25.9 ± 3.3 kg/m^2 (mean ± SD), were recruited for this study. The experiment was run for two consecutive days using the same microdialysis catheters CMA 66. Dialysate samples were collected as before. 2 ml of blood samples were collected using a cannula inserted into the anticubital vein. Samples were taken every hour for a period of five hours each day. The plasma and dialysate samples were analysed for adiponectin using the Mercodia kits. Adiponectin concentrations in plasma samples were 256 and 1791 times higher than the adiponectin concentrations in dialysate samples. The conclusion of the two studies was that the CMA 66 microdialysis catheter with cut off 100 KD membranes only recovers a small part of the total adiponectin present.

Therefore a third study was designed to use plasma samples. The aim of the study was to investigate the effect of acute exercise at 50% $\dot{V}O_{2\,max}$ on HMW adiponectin, total adiponectin, interleukin (IL)-6, tumour necrosis factor alpha (TNF-α), insulin and glucose concentrations directly after the exercise, one hour after and 48 hours. 13 young healthy female volunteers age 24.3 ± 2.7 years and BMI 21.9 ± 2.2 kg/m^2 (mean ± SD) contributed in this study. The volunteers were invited for five visits. Their fitness was measured on the first visit. Then they came for two main trials rest day and exercise day, which they were randomly assigned. The main trails lasted for two hours. Three blood samples were collected each day using same cannulated system in the second study. The volunteers followed 48 hours after each trial, one blood sample were collected each day. The 8 plasma samples were analysed for: total adiponectin and insulin concentrations via Mercodia ELISA kits, HMW adiponectin, IL-6 and TNF-α concentration via R&D systems and glucose concentration using the glucose oxidase colorimetric method.

The results showed no statistical difference in total or HMW adiponectin, TNF-α and glucose concentrations under the effect of moderate exercise at 50% $\dot{V}O_{2\,max}$ either directly or 48 (p value > 0.05). IL-6 concentrations increased about two fold one hour after the exercise above the resting level (P value < 0.05). IL-6 concentrations return to the basal level 48 hour latter. Insulin concentrations show a decrease one hour after the exercise finished. The number of volunteers was small and the change was close to significance. A one way ANOVA returned a P value of < 0.05, but a two way ANOVA with repeated measures returned a P value of > 0.05.

In conclusion, the acute exercise at 50% $\dot{V}O_{2\,max}$ changes IL-6 concentrations but it has no effect on adiponectin concentrations in dialysate or plasma samples. Low adiponectin concentration is related to obesity, insulin resistance and T2D. Therefore, increase in adiponectin concentration probably lies in weight loss and the exercise may play role, even if it has little direct action on adiponectin concentration.

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Dedication

To my parents, my sisters and my brothers I would like to show gratitude for being prayed for me all the time and for never ending love and encouragement.

To my dear husband, Hakeem ……, and my two lovely kids Mohamed & Omar. Yes Mohamed we can do it.

Mabrouka

Author's declaration

I declare that this thesis is of my own work and the experiments and results presented in it were made completely by me.

Mabroukah Mohamed Abubaker Alzwayi

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Definitions

µg	Microgram
µl	Micro litre
^{18}F-FDG	^{18}F-Fluorodeoxyglucose
Acrp 30	Adipocyte Complement Related Protein of 30 KD
Adipo R1	Adiponectin receptor 1
Adipo R2	Adiponectin receptor 2
ADSF	Adipose tissue specific secretary factor
Alb-LMW	Albumin Binding Trimmer
AMP	Adinosin Mono Phosphate
AMPK	Adenosine Mono Phosphate Kinase
ANOVA	Analysis of variance
apM1	Adipose most abundant gene transcript 1
ATP	Adenosine tri Phosphate
BMI	Body Mass Index
Ca	Calcium
CI	Confidence interval
Cl	Chloride
CMA	A Harvard Apparatus Company
CRP	C reactive protein
CVD	Cardio Vascular Disease
D	Diffusion coefficient
Da	Dalton
EDTA	Ethylene Diamine Tetra acetic acid
ELISA	Enzyme Linked Immune Sorbent Assay
FIZZ	Found in Inflammatory Zone
GBP-28	Gelatine Binding Protein -28
H_2SO_4	Sulphuric Acid
HDL	High Density Lipoprotein
HMW	High Molecular Weight
HMWA	High Molecular Weight Adiponectin
HOMA	Homeostatic model assessment
IL-6	Interleukin 6
IL-8	Interleukin 8
K	Potassium
KD	Kilo Dalton
kg	Kilogram

l	Litre
LMWA	Low Molecular Weight Adiponectin
MCP-1	Monocyte chemo attractant protein 1
MHR	Maximum Heart Rate
ml	Millilitre
mmol	Millimole
MMW	Middle molecular weight
mRNA	Messenger Ribo Nucleic Acid
mU	Micro unit
Na	Sodium
ng	Nanogram
NIDDM	Non Insulin Dependent Diabetes Mellitus
OD	Outer Diameter
PCO	Polycystic Ovary
PCr	Creatin phosphate
PET-CT	Positron emission tomography - computed tomography
pg	Pico gram
PKC	Protein Kinase C
RELMS	Resistin Like Molecules
RER	Respiratory exchange ratio
SAT	Subcutaneous Adipose Tissue
SCAAT	Subcutaneous Abdominal Adipose Tissue
SD	Standard deviation of mean
T2D	Type 2 Diabetes
TMB	Tetra Methyl Benzidine
TNF-α	Tumour Necrosis Factor Alpha
WAT	White Adipose Tissue
$\dot{V}O_{2\ max}$	Maximal oxygen consumption
$\dot{V}CO_2$	Carbon dioxide production

Chapter 1

Introduction and literature review

1.1 Introduction

Obesity has been the focus of increasing scientific and clinical interest for the last 30 years. This concern is justified because of its relationship to many serious diseases. The causes of obesity are varied and complex and the treatments may be complex too. Many researchers have focussed on exercise and diet as possible solutions. Therefore, it is timely to investigate the links between obesity, the responses to exercise and the metabolic diseases. This offers the hope that factors can be found which might improve our understanding.

Many authors have reported that adipose tissues are not only storage tissues, but also secrete a range of substances known as adipocytokines. These adipocytokines include leptin, resistin, TNF-α, IL-6, adipsin, visfatin and adiponectin. Some of these will be described later in section 1-3. The concentration of almost all these adipocytokines increases in obesity. The exception is adiponectin, whose concentration decreases in obesity, type 2 diabetes (T2D) and insulin resistance. This raises a question about why adiponectin concentrations decrease in obesity. Since adiponectin exerts several protective effects, it is obvious that it is a promising target for obesity treatments.

Adiponectin is a protein which is secreted mainly from the adipocytes. It is found in the circulation in a relatively high concentration ranging between 3 and 30 μg/ml. It is secreted as a monomer with molecular weight of 30 KD. These form trimers and hexamers and multimers of high molecular weight (HMW). The most common forms found in the plasma are the hexamers and HMW. The HMW form has the greatest biological activity.

Low concentrations of adiponectin have been associated with several metabolic diseases, particularly T2D and cardiovascular disease (CVD). One

function of adiponectin is to enhance insulin sensitivity and low adiponectin concentrations have been suggested to play a role in the development of insulin resistance. Administration of adiponectin has been found to reduce the glucose concentration in plasma and improve insulin sensitivity. Thiazoledinedione is used in the treatment of T2D. Thiazoledinedione increases adiponectin concentrations in plasma and that might be related to the effect of thiazoledinedione on insulin concentration. In addition, many studies have reported a relationship between low concentrations of adiponectin and liver fibrosis and cancer.

It is well known that exercise increases insulin sensitivity. Adiponectin plays a role in sensitizing tissues to insulin. Obesity is associated with insulin resistance. Thus, what is the link between exercise and insulin sensitivity. Since adipocytes secrete adiponectin, is there any role for adiponectin in increasing insulin sensitivity in exercise.

Collection of adiponectin at its site of production is of special interest. Microdialysis is a relatively new technique which has been used to collect molecules from the extracellular space of many different tissues, including adipose tissue. This technique can be used to sample fluid composition continuously for up 4 days. Recently, microdialysis has been established for monitoring of adipokines and cytokines in the extracellular fluid of subcutaneous adipose tissue of human in vivo.

Obesity, particularly visceral obesity, is more common in females than in males. Interestingly, adiponectin concentrations in plasma samples tend to be higher in females than males. However, most of the studies on the effect of exercise on adipocyte secretions study males. Again this raises questions about how similar the responses to exercise are in women and how does the increased obesity in women affect the responses.

Literature Review

1.2 Obesity

Obesity is most commonly defined as a Body Mass Index (BMI) of 30 kg/m^2 or above. It is very big health problem, and it is associated with many diseases such as diabetes, coronary heart disease and hyperlipidaemia (Lean, 2000). Recently, a national survey based in the USA has shown that the prevalence of being overweight or obese among adults remained relatively constant between 1960 and 1980. The prevalence began to increase in the mid-1980s and it has continued to increase since then (Flegal *et al.*, 1998). It is about 20% in USA and it is higher in black than the white population (Flegal *et al.*, 1998). A similar picture is seen in Europe. The prevalence of obesity in Britain had increased three times from 1980 to 1997 (Kopelman, 2000).The prevalence in the UK in 2002 had increased to 23% and 25% for males and females respectively(Department of Health, 2004).

The picture is similar in Arab populations. This figure was similar to that found in Morocco, Tunisia (Mokhtar *et al.*, 2001). One study in Saudi Arabia found a prevalence of obesity of 26% in males and 44% in females (Al-Nozha *et al.*, 2005). There may be variation with the Arab world because a lower percentage of 16% was found among females in United Arab Emirates (Sheikh-Ismail *et al.*, 2009). All studies demonstrate that the prevalence of obesity is higher among females than males.

Moreover, this problem extends to include children. Data from the UK showed the prevalence of obesity among 15 year old children was 17%, (Reilly & Dorosty, 1999). However, this percentage increased in 2004 to 24% for males and 26% for females (Reilly, 2006). In the USA Ogden et al, found that the prevalence of overweight and obesity among young males 2-19 years old was 31.9% and 11.3% (Ogden *et al.*, 2008).

The trend is found among children in Arab countries. Similar results have been found in Kuwait (Al-Isa, 2004), Qatar (Bener, 2006) and The United Arab Emirates (Malik & Bakir, 2007). In Saudi Arabia the prevalence was

transitional between developing and developed countries, 20.4%, overweight and 5.7% obese respectively (El-Mouzan *et al*, 2010).

There are regional variations, in Yemen the prevalence was low at 6.2% and 1.8% for overweight and obesity among schoolchildren respectively (Raja'a & Bin Mohanna, 2003). This pattern could reflect economic conditions.

Furthermore, the prevalence of overweight is similar among girls and boys. In contrast, the prevalence of obesity and severe obesity is higher in boys than girls (El-Mouzan *et al.*, 2010). The level of education has an impact on this problem. It has been found that the obesity rates are lower in more educated groups (Pi-Sunyer, 2002). Figure 1-1 shows the prevalence and absolute numbers of overweight and obesity in many regions in the world. These data are for adults and show the position in 2005. Across the whole world, the study suggests that 9.8% of the population are obese with females have higher prevalence of obesity than males. This percentage represents 396 million people. The prevalence of obesity is rising steeply and the prediction is that it will >55% in 2030 if the recent trends continue (Kelly et al., 2008). The data show considerable regional variations and this probably reflects many different causes of obesity. It is unlikely there will be a single solution for all cultures.

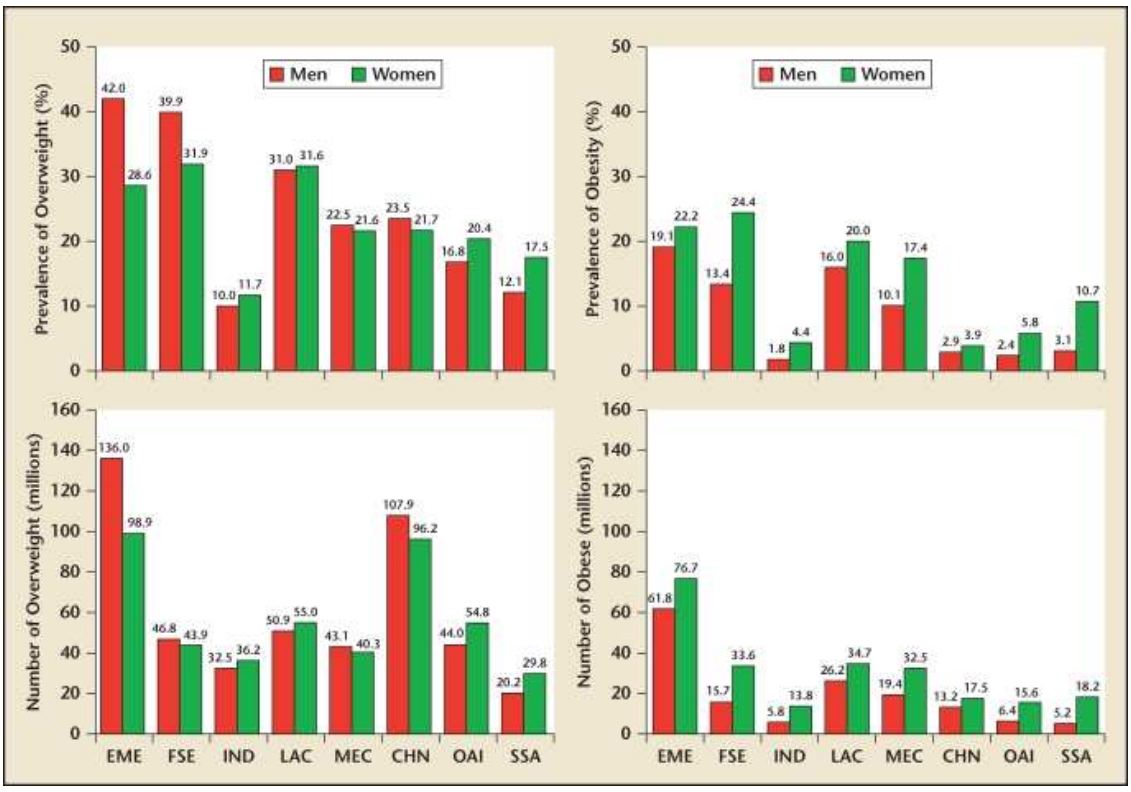


Figure 1-1

The prevalence (upper) and absolute number (lower) of overweight and obesity in adults in different regions for both males and females in 2005. CHN, China; EME, established market economies; FSE, former socialist economies; IND, India; LAC, Latin America and the Caribbean; MEC, middle eastern crescent; OAI, other Asia and islands; SSA, sub-Saharan Africa. Reprinted with permission from (Kelly *et al.*, 2008)

1.2.1 Causes

Obesity is associated with a heterogeneous group of conditions with several causes. The interactions between genetic, ecological and behavioural factors are responsible for a progressive rise in body weight. The last two factors are probably mostly responsible for the prevalence of obesity, but also heredity factors play an important role (Kopelman, 2000). These factors will be covered individually.

1.2.1.1 Genetic factors

The variation in prevalence of obesity worldwide has led to an impression that heredity may play an important role in determining how common obesity is in each society. There is considerable evidence to suggest that height and weight are heritable traits (Barsh *et al.*, 2000). Overall, data

from twin and adoption studies give a consistent finding showing that genetic factors contribute between 40% and 70% of the variation in BMI (Maes *et al.*, 1997). Other studies of the causes of obesity estimate the percentage contribution of genetic factors from 30% to 40% and environmental factors between 60% and 70% (Pi-Sunyer, 2002). Several heritable factors are likely to be responsible and are likely to work through various possible mechanisms, such as energy intake and energy expenditure (Farooqi & ORahilly, 2007). It is also important to consider the interaction between genetic and environmental factors such as availability and nutritional content of food. For instance, in some cases the expression of genotype needs certain adverse environmental conditions (Stunkard, 1988).

1.2.1.2 Environmental factors

Obesity develops when energy intake is more than energy expenditure. Nowadays, lifestyle in developed countries is associated with the availability of convenient, cheap food with a high energy content and increasingly with sedentary life style requiring less physical activity (Hill & Peters, 1998). The low level of exercise or physical activity is directly linked to obesity. In Finland, a decline in physical activity at work and in transport during the past 10 years has been accompanied by a significant increase in leisure time (Kopelman, 2000). It has been demonstrated that decrease in energy expenditure results in an increase weight seven times (Ferro-Luzzi & Martino, 1996).

These trends may be more extreme for women than for men. Almost all studies show that females are more obese than males (El Ati *et al.*, 2012; Flegal *et al.*, 2010; Kelly *et al.*, 2008). This effect is clearly seen in some Arab countries. The reduction in the level of activity by women might be very large because there is an increasing trend to employ housemaids and there is a culture which makes it difficult for women to exercise (Alsaif *et al.*, 2002).

In the case of children, the introduction of electronic devices, such as computers, televisions and electronic games, allow children spend much

time in sedentary activities. This might contribute to the high prevalence of obesity in children (Kautiainen *et al.*, 2005). Several studies have shown a positive relation between time spent watching television and the incidence of obesity in children (Kautiainen *et al.*, 2005; Lioret *et al.*, 2007). In contrast, Kautiainen found that being overweight was not related to time spent playing computer games (Kautiainen *et al.*, 2005).

1.2.2 Fat distribution

As described above in section 1.2, obesity is a problem almost everywhere in the world. The form of body shape associated with obesity depends on the degree and site of the fat accumulation. The regional distribution of fat may be extreme in some ethnic groups, like Hottentot women, where the fat is accumulated in their buttocks (Ersek *et al.*, 1994). The accumulation of adipose tissue in humans takes place over an extended time period. Body fat is a very specific tissue that stores metabolic energy in the form of triglycerides and releases them when the other tissues in the body need energy (Cinti, 2007). It is mainly found either superficially in subcutaneous stores or more deeply in visceral stores. In lean male adults, adipose tissue constitutes about 8 to 18% of body weight, while for females it is about 14 to 28%. This percentage greatly increase in case of obesity and could reach 60 to 70% of body weight (Cinti, 2007).

The adipose tissue pool has at least two functionally different types of fat: white and brown adipose tissue. In general, accumulation of white adipose tissue is associated with increased risk of diseases, while the brown adipose tissue reduces the risk of obesity and is associated with a protective action (Chalfant *et al.*, 2012).

1.2.2.1 White adipose tissue

White adipose tissue (WAT) is the primary site for the fat storage. It also secretes hormones and releases cytokines (Ronti *et al.*, 2006). About 30 % of circulating IL-6 comes from WAT mainly from visceral adipose tissue (Fried *et al.*, 1998).

WAT shows clear differences between genders. Women have lesser amounts of intra-abdominal white adipose tissue and more subcutaneous white adipose tissue (SAT). In women SAT is found more in the thigh and hip regions than men (Gesta *et al.*, 2007). Heredity can also have a big influence on fat mass and fat distribution (Montague & O'Rahilly, 2000). In addition, Saxena et al found that the ethnic group to which children belonged was more important than their social class in determining their chances of being overweight or obese (Saxena *et al.*, 2004). They found that British Afro-Caribbean and Pakistani girls and Indian and Pakistani boys have a significantly increased risk of being overweight or obese than the general population (Saxena *et al.*, 2004).

Obesity occurs as a result of WAT accumulation. It is most dangerous if it is in the visceral area because of its correlation with metabolic risk factors like high blood pressure, dyslipidemia, insulin resistance and T2D (Briatore *et al.*, 2008) and cardiovascular disease (Fox *et al.*, 2007). The risk is lower if this accumulation is subcutaneous around the gluto-femoral region or in the lower part of the abdomen (Arner, 1998; Haffner *et al.*, 1991; Lakka *et al.*, 2002). Not only this but also extra fat could be stored in other fat deposits around and within other tissues and organs such as heart, blood vessels and kidney, which is known as “ectopic fat storage”. This accumulation of fat around such organs could impair their functions (Montani *et al.*, 2004). The fat immediately adjacent to blood vessels may be one key factor predisposing to complications in obesity. The mobilisation of free fatty acids from visceral adipose tissue is more rapid than from SAT due to the higher lipolytic activity in visceral adipocytes. This variation involves the function of different andrenergic adrenoceptor subtypes and the function of insulin receptors (Arner, 1998).

Adiponectin influences many cellular processes in the heart and vascular endothelial cells and these processes are mediated by adenosine mono phosphate kinase (AMPK) activation. In endothelial cells the signalling of AMPK is required for the adiponectin proangiogenic effects (Ouchi *et al.*, 2004; Shibata *et al.*, 2004) and reduction of myocardial infarct size in a mouse model of heart ischemia (Shibata *et al.*, 2005).

1.2.2.2 Brown adipose tissue

Brown adipose tissue is mainly found in infants and young children (Cannon & Nedergaard, 2004). In new born infants it is important for thermo-genesis and it affects whole body metabolism. It may also alter insulin sensitivity (Almind *et al.*, 2007). However, there is a continuing debate about the presence of brown adipose tissue normally in adults (Garruti & Ricquier, 1992). Significant masses of brown adipose tissue have been found in adult males and females using ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron-emission tomographic and computed tomographic (PET-CT) scans (Cypess *et al.*, 2009). Brown adipose tissue has been found to be active in adults exposed to cold conditions or subjected to hyper-adrenergic stimulation in pheochromocytoma (Nedergaard *et al.*, 2007). The distribution of brown adipose tissue is similar in male and females, however; the accumulation and activity of brown adipose tissue are more in females than males (Cypess *et al.*, 2009).

1.2.3 Problems

Many physiological and psychological problems are associated with obesity. These include diabetes, cardiovascular diseases, hypertension, cancers, joint trauma, increased mortality, low self esteem and depression (Ashton *et al.*, 2001; Wadden *et al.*, 2006).

1.2.3.1 Insulin Resistance and Hyper-insulinaemia

Insulin is an important hormone in metabolic regulation. It is synthesised by the ß cells of the pancreas. It stimulates glucose up take in many organs, especially muscle, liver and adipose tissue. Insulin also inhibits lipolysis in adipose tissue (Ruan & Lodish, 2003). Insulin resistance can be inherited or it can be acquired as a consequence of obesity (Pi-Sunyer, 2002). One of the proposed mechanisms of insulin resistance which is induced by lipid is the impairment of insulin action either through stimulation of protein kinase C (PKC) activity or directly (Hegarty *et al.*, 2003). Kahn et al. showed that insulin resistance is directly proportional to BMI in non-diabetic individuals

(Kahn et al., 1993). Furthermore, in normal and overweight females the main determinant of insulin resistance is visceral obesity. This is a stronger influence than total body fat content, limb or trunk fat (Carey *et al.*, 1996). Peripheral obesity may even protect against the development of insulin resistance (Lewis *et al.*, 2002).

There are two theories proposed to explain how visceral obesity induces insulin resistance. Firstly, the expansion of adipose tissue ultimately saturates its storage capacity and therefore limits its ability to accumulate more fat. Consequently, the excess fat will accumulate in other organs such as the liver, pancreas and muscles. The accumulation of fat on these organs can induce insulin resistance. Secondly, the extra accumulation of fat in adipose tissue is able to alter its ability to secret adipokines such as adiponectin, leptin, resistin, IL-6 and TNF-α. These adipokines regulate insulin sensitivity in adipose tissue and other organs (Fantuzzi, 2005).

One of the most important pathogenic features of T2D is insulin resistance (De Leo *et al.*, 2003). Therefore, insulin resistance should be managed to treat these metabolic disorders. The most effective treatments for insulin resistance are weight loss and exercise (Greenfield & Campbell, 2004). Pharmaceutical approaches are also used. Metaformin and thiazolidinediones can have a very effective role in treatment of insulin resistance (Greenfield & Campbell, 2004). The mode of action of metaformin is through the reduction of hepatic glucose production (Inzucchi *et al.*, 1998). It also decreases the systemic concentration of triglyceride and free fatty acids (Bailey & Turner, 1996). While the precise mechanism of thiazolidinediones action is not clear, part of its action is thought to be through effects on body fat (Gurnell *et al.*, 2003)

1.2.3.2 Type 2 Diabetes and obesity

T2D is also known as non insulin dependent diabetes or diabetes mellitus (NIDDM). It develops in two ways: as a result of insulin resistance where there is a failure of the body to respond to insulin, or by a decrease in the ability of the pancreas to secret an adequate amount of insulin (Banting *et*

al., 1922; Garvey *et al.*, 1985). This form of diabetes is usually associated with obesity. It is now very common (Mokdad *et al.*, 2003).

Many studies have found a close link between diabetes and obesity (Colditz *et al.*, 1995; Ford *et al.*, 1997; Wannammethee & Shaper, 1999). The development of diabetes increases gradually with increasing BMI for males and females. Colditz et al. found that the risk of diabetes in adult females increases two fold if body weight increases by 7 kg. The risk of developing diabetes is four times greater if their BMI is more than 23 kg/m^2 than it is if the BMI less than 22 Kg/m^2 (Colditz *et al.*, 1995). Koh-Banerjee et al. determined that the risk of developing diabetes increases by 7.3% for every kilogram weight gained (Koh-Banerjee *et al.*, 2004). They also found a close relation between fat distribution and the risk of T2D. Abdominal obesity might be stronger predictor of risk of diabetes than BMI alone (Hartz *et al.*, 1983; Koh-Banerjee *et al.*, 2004; Ohlson *et al.*, 1985). The bigger size of the abdominal adipocytes might be related with metabolic disturbance. In addition, the abdominal adipocytes are more responsive to insulin than the femoral adipocytes (Smith *et al.*, 1979).

1.2.4 Possible Solutions to the problem of obesity

The National Weight Control Registry has pointed out that diet and exercise are the bases in the treatment of obesity (Wang et al., 2002). Furthermore, it has been demonstrated that exercise is an important component of control of weight and that a higher duration and intensity of exercise may improve long term weight loss. Weight loss also has beneficial effects which reduce the health risks (Jakicic & Otto, 2005). In short, adipose tissue, in addition to being specialized organ for fat storage, can be considered as the largest endocrine gland because it synthesises and secrets a range of hormones known as adipokines many of which affect insulin sensitivity.

1.3 Adipose tissues as an endocrine organ

WAT is not only a storage organ but also complex endocrine organ. It secretes free fatty acids, cytokines and a range of hormones known as

adipokines. They are peptides or small proteins secreted by adipocytes into the systemic circulation and they have autocrine, paracrine and endocrine actions (Antuna-Puente *et al.*, 2008; Bastard *et al.*, 2006; Hill *et al.*, 2009)

Many biological functions in many organs, including the brain, liver, adipose tissue, skeletal muscles, reproductive system and blood vessels are related to adipokines (Hill *et al.*, 2009). These adipokines include: leptin, adiponectin, resistin, visfatin, adipsin, IL-6 and TNF- α (Antuna-Puente *et al.*, 2008). For instance, adipokines play a vital role in the regulation of glucose and lipid metabolism because they enhance insulin sensitivity (Fisher *et al.*, 2005; Oh *et al.*, 2007; Scherer *et al.*, 1995). Adipokines affect lipid intake and energy expenditure (Ahima & Flier, 2000). They have an anti atherogenic and anti inflammatory action (Fisher *et al.*, 2005; Oh *et al.*, 2007; Scherer *et al.*, 1995). Eventually, the over production of these adipokines is linked with some pathological conditions relating to lipid metabolism, insulin sensitivity and CVD (Hill *et al.*, 2009).

Adipose tissue is responsible for changes in the physiological and functional role of these adipokines in the body. However, these adipokines are also present in other parts of the body such as the immune system, reproductive system, brain, liver and skeletal muscle (Hill *et al.*, 2009). The secretion of adipokines show signs of regional variation, where the release and expression of IL-6 and IL-8 are more expressed and released by visceral adipose tissue than subcutaneous adipose tissue (Fried *et al.*, 1998). While adiponectin and leptin are more expressed and released by subcutaneous adipose tissue than visceral adipose tissue (Lihn *et al.*, 2004).

1.3.1 Leptin

Leptin is one of the best known adipokines. It is a 16 KD protein made up of 146 amino acids and it is encoded by the *ob* gene (Zhang *et al.*, 1994). It was named *leptin* from Greek word meaning ‘thin’ (Ahima & Flier, 2000). There is a direct relationship between leptin concentration and obesity; leptin concentrations are higher in obese people than in lean people (Minokoshi & Kim, 2002). While this adipokine is thought to be mainly expressed by white

adipose tissue, recent research has showed that it is also expressed in many other sites including the hypothalamus (Morash *et al.*, 1999), the pituitary gland (Jin *et al.*, 2000) and skeletal muscle (Wang *et al.*, 1998).

Leptin has several actions. It enhances insulin sensitivity by decreasing the fat concentration in skeletal muscle, liver and pancreatic beta cells (Ronti *et al.*, 2006). Another important action, it influences the control of food intake and energy expenditure by the central nervous system (Ahima & Flier, 2000). There is strong evidence to show that the main action for leptin is to act as a 'starvation signal'. In addition its concentrations in plasma and adipose tissues are dependent on the amount of the energy stored. The concentration of leptin increases in case of over feeding and decrease in fasting. Consequently; it is higher in obese individuals and lower in lean individuals (Ahima & Flier, 2000). Leptin concentrations increase in response to hyperinsulinaemia, glucocorticoids, acute infections and proinflammatory cytokines. On the other hand, leptin concentrations are decreased in response to the stimulation of thyroid hormone, growth hormone and thiazolidinediones (Ahima & Flier, 2000).

1.3.2 Resistin

Resistin is another important hormone of this group. It is also known as "Adipose tissue-specific Secretary Factor" (ADSF) (Kim *et al.*, 2001) and as "found in inflammatory zone" FIZZ (Holcomb *et al.*, 2000). Resistin is a peptide hormone of 12.5 KD. It is composed of 114 amino acids and is encoded by a gene known as retn. This gene is found on chromosome 19 in humans and on chromosome 8 in mice. It is belonging to group of proteins rich with cysteine named "resistin – like molecules" (RELMs) (Rajala *et al.*, 2003).

Resistin is found to induce insulin resistance in mice (Rajala *et al.*, 2003). Its production in adipose tissue is suppressed by thiazoladinedione treatment. It is believed that resistin could be the link between obesity and insulin resistance (Steppan *et al.*, 2001). Resistin is similar to all adipokines, except adiponectin, in its relationship to obesity. It has a direct relationship with

obesity (Rajala *et al.*, 2003). The greater part of the study of resistin actions has been done in mice. The physiological role of resistin in humans is not clear (Savage *et al.*, 2001).

1.3.3 Tumour Necrosis Factor α (TNF-α)

TNF–α was the first molecular link between obesity and insulin resistance to be identified (Moller, 2000). TNF-α is a pro-inflammatory cytokine, secreted mainly by lymphocytes, macrophages. Adipocytes in humans produce small amounts of this hormone (Hotamisligil *et al.*, 1993). It is 26 KD protein with 157 amino acids, composed of three identical subunits (Hotamisligil *et al.*, 1993).

TNF-α mRNA shows a relationship with BMI, percentage of body fat and hyper-insulinaemia. Its concentration decreases during weight loss and after improvement of insulin sensitivity (Guerre-Millo, 2004). Insulin signalling is inhibited by TNF–α and this might be the mechanism of insulin resistance in obesity (Fried *et al.*, 1998). In addition, adiponectin secretion from adipocytes is inhibited by TNF–α (Garg, 2004). In contrast, Ofei et al. demonstrated that glucose concentration and insulin sensitivity were not altered by the infusion of antibodies raised against TNF–α (Ofei *et al.*, 1996).

The role of TNF–α and IL-6 in the regulation of adiponectin secretion has been studied by many researchers. Maeda et al. confirmed the inhibitor role of TNF-α on adiponectin production and gene expression (Maeda *et al.*, 2001). In humans, TNF-α has been shown to decrease adiponectin gene expression and adiponectin secretion from adipose tissue (Lihn *et al.*, 2003b). The same result has been found in pre-adipocytes (Kappes & Loffler, 2000). These and other data from in vivo studies in humans and rodents show a negative relation between TNF-α and adiponectin (Lihn *et al.*, 2003b). As a result, TNF–α might play an important role in the path-physiology of insulin resistance (Hotamisligil *et al.*, 1993).

1.3.4 Interleukin – 6 (IL-6)

IL-6 is a cytokine with many forms, ranging from 22 to 27 KD. It is secreted by many cells including: fibroblasts, endothelial cells and monocytes. Adipose tissues secrete significant amounts of IL-6, about 10% of IL-6 is secreted from adipocytes themselves (Fried *et al.*, 1998). As with other adipocytokines, the IL-6 concentrations increase in obesity, particularly visceral obesity. The expression of IL-6 mRNA is higher in visceral adipose tissue than subcutaneous adipose tissue (Fried *et al.*, 1998). IL-6 secretion is related to BMI and fat mass (Vozarova *et al.*, 2001). There is a low concentration of IL-6 mRNA in resting muscle but IL-6 is secreted by skeletal muscle in response to exercise (Plomgaard *et al.*, 2005).

IL-6 has many functions. One of the most important is the stimulation of C reactive protein (CRP) production in the liver (Ridker, 2003). Moreover, IL-6 is an important link between obesity, inflammation and coronary heart disease (Yudkin *et al.*, 2000). Other studies have suggested that IL-6 might play a role in insulin resistance (Bastard *et al.*, 2002). In addition, IL-6 inhibits adiponectin secretion and decreases the signals of insulin receptors (Kershaw & Flier, 2004).

IL-6 is one of the cytokines which increases in obesity and this might be responsible for the high level of acute-phase proteins, such as CRP, in obese subjects (Fantuzzi, 2005).

1.3.5 Adiponectin

Adiponectin is one of the main adipokines. It has several names: “adipocyte complement-related protein of 30 KD” (Acrp30) (Scherer *et al.*, 1995), “Adipose most abundant gene transcript 1” (apM1) (Maeda *et al.*, 1996), “gelatine binding protein -28” (GBP28) (Nakano *et al.*, 1996) or adipo Q (Hu *et al.*, 1996). These five names were given by the five different research groups who worked on the early stage of its detection. Scherer was the first to identify adiponectin as a protein synthesized in adipose tissue and secreted from mouse adipocyte cells from the 3T3-L1 line. At about the

same time, Nakano et al. discovered a similar protein with a molecular weight of 28 KD. Maeda et al. isolated the specific gene in adipose tissue from humans and called it apM1. A polypeptide of 247 amino acids was found by Hu et al. working on adipose tissue using cDNA and mRNA techniques (Hu *et al.*, 1996)

Adiponectin is secreted mainly by adipocytes; however, it can be expressed by osteoblasts (Berner *et al.*, 2004), skeletal muscle (Krause *et al.*, 2008) cardiomyocytes (Pineiro *et al.*, 2005) and epithelial cells in salivary glands (Nishida *et al.*, 2007). It circulates in plasma in relatively high concentrations of about 3- 30 µg/ml (Arita *et al.*, 1999; Scherer *et al.*, 1995). It accounts for about 0.01% of the total plasma proteins in normal persons (Antuna-Puente *et al.*, 2008; Whitehead *et al.*, 2006). Thus the concentration of adiponectin is far higher than the concentration of any other hormone in the blood of lean individuals (Hu *et al.*, 1996; Maeda *et al.*, 1996; Nakano *et al.*, 1996).

This concentration of adiponectin in plasma is three times higher than the concentration of leptin, which is typically measured as nanograms per millilitre, and about six times the concentration of IL-6, which is typically measured as picogram per millilitre. One question arises at this point: why is the concentration of adiponectin so much higher than other hormones secreted by adipose tissue? One might speculate that it reflects its important role. Alternatively, it might exert its action through very low efficiency receptors (Sun *et al.*, 2009).

1.3.5.1 Structure

Adiponectin is similar in structure to complement protein C1q and tumour necrosis factor α Figure 1-2. It consists of four domains including amino terminal region, a variable region, a globular domain near the c terminus, and a collagen-like domain (Scherer *et al.*, 1995). Total adiponectin acts to reduce glucose production in the liver (Combs *et al.*, 2001). In contrast, the globular form of adiponectin plays an important role in stimulation of fatty acid oxidation in liver and skeletal muscle (Yamauchi *et al.*, 2003a).

Therefore, the way in which the different forms of adiponectin work and their site of the action might be different (Wang *et al.*, 2002).

1.3.5.2 Forms

There are many isoforms of adiponectin found in human blood and each may have different effects (Hill *et al.*, 2009). These forms are illustrated in figure 1-3. The principal forms are: low molecular weight probably a trimer (Mangge *et al.*, 2009), the albumin binding trimer (Alb-LMW), middle molecular weight or hexamer (MMW) and a high molecular weight form, which probably consists of large multimers (Hada *et al.*, 2007; Hill *et al.*, 2009).

These forms have been identified by a range of different techniques such as gel filtration chromatography, velocity sedimentation centrifugation and reducing polyacrylamide gel electrophoresis (Oh *et al.*, 2007)

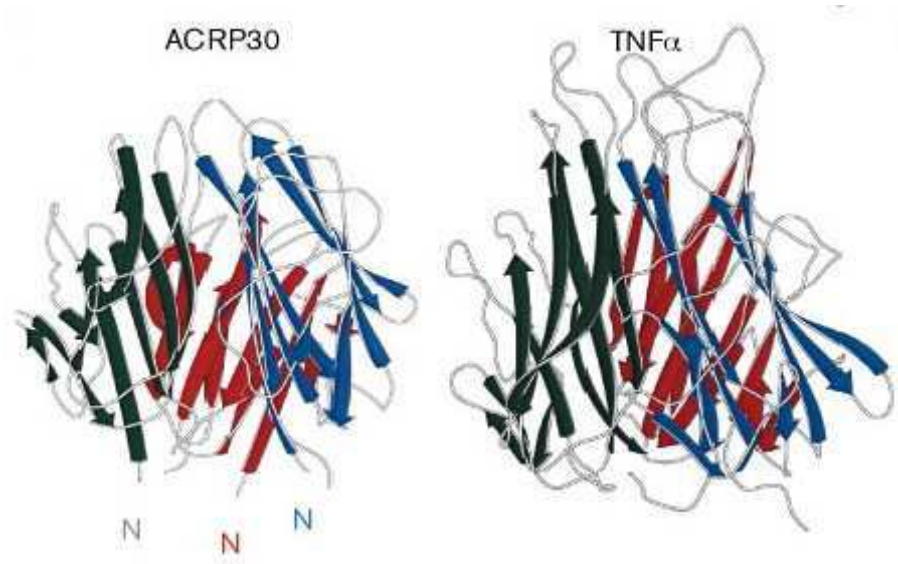


Figure 1-2

Adiponectin structure and it is similarity with TNF-α (Shapiro & Scherer, 1998)

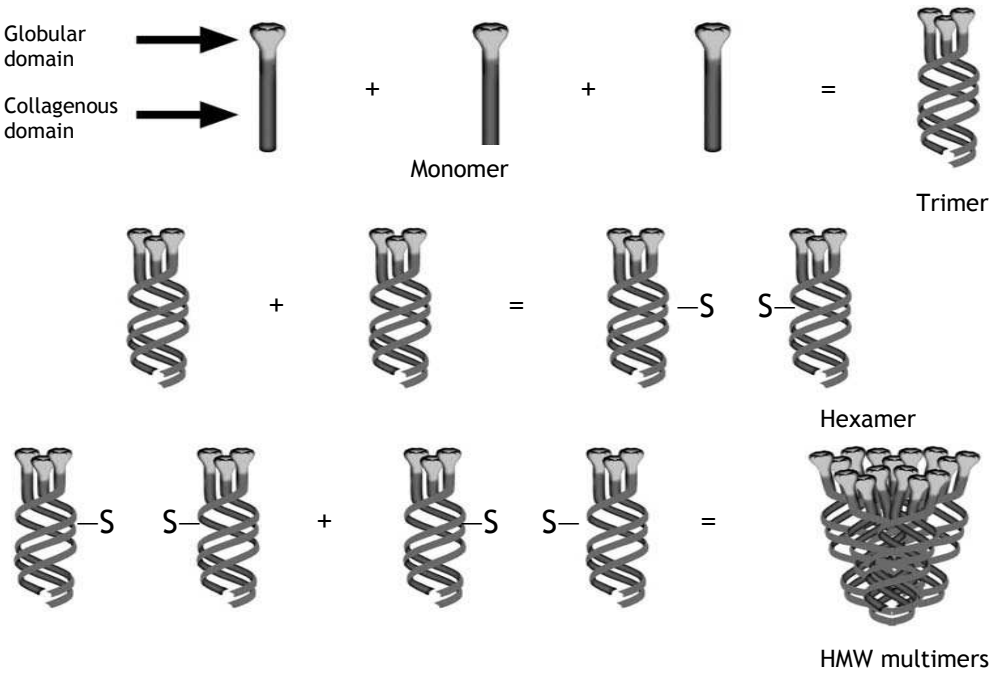


Figure 1-3

Multimeric forms of adiponectin. (Oh *et al.*, 2007)

The concentrations of many adipokines are significantly different in plasma and interstitial fluid. The concentration of adiponectin and leptin in interstitial fluid is lower than in plasma (Nielsen *et al.*, 2009). This is surprising because the main site for adiponectin secretion is adipose tissue. There are two possible explanations for this. Firstly, adiponectin may be transported actively from the secretion site to plasma. Secondly, adiponectin in plasma found in two forms, a free form and a bound form which binds strongly to a transporter protein. The low concentration of the free form may be in equilibrium with interstitial fluid. Furthermore; the distribution of adipose tissue around the body is clinically important. Accumulation of fat in abdominal regions is more dangerous than elsewhere since insulin resistance is related to the accumulation of abdominal adipose tissue. In contrast, lower –body fat mass is related positively to insulin sensitivity (Buemann *et al.*, 2005). Cnop et al. showed that, there is a positive relation between adiponectin concentrations and insulin sensitivity. This supports the concept that there is a link between adiponectin and insulin action. However, the concentration of adiponectin is negatively related to the distribution of body fat and the size of the body. Further there is a strong correlation between adiponectin concentration and the

concentration of HDL cholesterol and the size of LDL particle. Another finding in this study is that the relation between adiponectin and obesity is similar in both sexes (Cnop *et al.*, 2003).

Adiponectin does not circulate as monomer, but it forms multimeric structures through disulphide bonds that link the collagenous domains (Trujillo & Scherer, 2005). This is illustrated in figure 1-3.

The monomeric form of adiponectin is found only in adipose tissue but not in the peripheral circulation (Giannessi *et al.*, 2007). Ebinuma et al. reported that 50% of the total adiponectin was in the HMW form and 25% in each of the MMW and LMW forms (Ebinuma *et al.*, 2006). Beltowski et al. Found slightly different proportions: HMW were 40%, MMW 35% and LMW 25% of the total adiponectin in human serum (Beltowski *et al.*, 2008).

The trimer is the basic form of circulating adiponectin (Wang *et al.*, 2006). These trimers range between 75-90 KD and can join together to form low molecular weight hexamers between 180 – 190 KD (Mangge *et al.*, 2009) and high molecular weight forms, with molecular weight of more than 420 KD (Navaneethan *et al.*, 2010). The LMW and HMW are more easily detectable in blood than the trimer. This is probably because the trimer has a the shorter half life (Pajvani *et al.*, 2003b). The HMW form is only formed in eukaryotic cells, but the maximum form found in the prokaryotic cells are the trimer and hexamer (Richards *et al.*, 2006).

Many authors report that HMW adiponectin is the most active form and its concentration is changed significantly with some diseases (Fisher *et al.*, 2005; Hara *et al.*, 2006; Hill *et al.*, 2009; Tsao *et al.*, 2003). For example, low concentrations of HMW are found in patients with T2D (Hill *et al.*, 2009). In addition, the effect of insulin resistance on adiponectin secretion is mainly because of the down regulation of HMW multimers (Basu *et al.*, 2007). Furthermore, the proportion of HMW is higher in women than men (Pajvani *et al.*, 2003a). HMW adiponectin is a better predictor of insulin resistance, metabolic syndrome (Fisher *et al.*, 2005; Hara *et al.*, 2006) and

coronary artery disease (Von *et al.*, 2008). Little data has been published about the physiological role of the multimeric forms of adiponectin.

1.3.5.3 Secretion

The production of adiponectin by adipocytes is a multistep process. This process of production can be regulated at the level of gene expression, secretion and the formation of the isoforms of the adiponectin (Guerre-Millo, 2008). The molecular mechanisms of adiponectin secretion are unknown (Motoshima *et al.*, 2002; Qiang *et al.*, 2007; Guerre-Millo, 2008). Adiponectin is synthesized as a single polypeptide of 30 KD and before its secretion it is assembled to form HMW in the endoplasmic reticulum (Qiang *et al.*, 2007). Adiponectin has been shown to be secreted from human visceral adipose tissue in vitro, and its mRNA appears to be negatively regulated by glucocorticoids and positively by insulin (Halleux *et al.*, 2001). Hajri et al suggested the requirement of insulin sensitivity for adiponectin secretion (Hajri *et al.*, 2011). Other studies have demonstrated that adiponectin expression and secretion from adipose tissue are regulated by insulin, TNF- α and IL-6 (Fasshauer *et al.*, 2002; Fasshauer *et al.*, 2003). Further more, endothelin-1, which is an important vasoconstrictor, has a role in the stimulation of adiponectin secretion from the 3T3-L1 adipocytes. However its mechanism of action on adiponectin secretion is unknown (Clarke *et al.*, 2003).

Motoshima et al demonstrated that the adiponectin secretion was not different between the omental and subcutaneous adipose tissue; however the stimulation of the secretion was different. Adiponectin is secreted more from omental adipose cells than subcutaneous cells under the effect of insulin. The basal adiponectin secretion from omental adipose tissue was negatively correlated to BMI, while there was no correlation between the secretion from subcutaneous adipose tissue and BMI (Motoshima *et al.*, 2002).

1.3.5.4 Regulation

Adiponectin has a relatively long half life of 2.5-6 hours in human plasma (Hoffstedt *et al.*, 2004). Its concentration shows some diurnal variation in human subjects as it is higher in the late morning and lower at night (Gavrila *et al.*, 2003). "Ultradian pulsatility'' has been observed in circulating adiponectin levels (Gavrila *et al.*, 2003). It is remarkable that transcription, translation and post translation levels of adiponectin may take part in its regulation. Moreover, variations in the renal and hepatic clearance of adiponectin provides another mechanism for regulation of its concentration (Tietge *et al.*, 2004).

Adiponectin is known to be regulated by other adipocytokines such as TNF-α and IL-6. The expression and secretion of adiponectin from human pre-adipocyte is decreased by TNF-α (Kappes & Loffler, 2000). Weight loss has a direct effect on adiponectin concentration. However, weight loss induced by calorie restricted diet does not affect adiponectin concentrations (Garaulet *et al.*, 2004; Xydakis *et al.*, 2004).

1.3.5.5 Distribution

Adiponectin concentrations vary. It is higher in plasma than in the interstitial fluid of subcutaneous adipose tissue in both lean and overweight males (Hojbjerre *et al.*, 2007). Its gene expression is higher in subcutaneous adipose tissue than visceral adipose tissue (Lihn *et al.*, 2004), but there is no difference between the concentrations in interstitial fluid collected from the gluteal and abdominal subcutaneous adipose tissue (Nielsen *et al.*, 2009)

Adiponectin concentrations are also affected by factors such as gender, age and ethnicity (Hill *et al.*, 2009). Women have higher adiponectin concentrations than men (Cnop *et al.*, 2003; Gilardini *et al.*, 2006). This is probably due to the difference in fat distribution, since women have larger subcutaneous fat deposits and the inhibitory effects of androgens on adiponectin secretion in men (Cnop *et al.*, 2003). Nishizawa et al demonstrated that the secretion of adiponectin was suppressed by

testosterone and dihydrotestosterone (5α-DHT) in 3T3-L1 adipocytes. This adiponectin concentration in plasma of mice treated with androgens was also reduced (Nishizawa *et al.*, 2002). There is no clear view of the effect of oestrogens. It has been suggested that the oestrogens could have a stimulatory effect on the production of adiponectin (Sieminska *et al.*, 2005). However one investigation found negative effects of oestrogens on adiponectin concentrations (Combs *et al.*, 2003).

Adiponectin concentration is affected by age and it is higher in older people than young people. This finding may be related to changes in testosterone and oestrogens concentrations with increasing age, possibly removing the inhibitory effect (Nishizawa *et al.*, 2002). Another possible reason is the reduction of adiponectin clearance in older people (Cnop et al. 2003).

Adiponectin concentrations are affected by the ethnicity. Its concentration is lower in Indo-Asians and Black African people than Caucasians people (Steffes *et al.*, 2004).

1.3.5.6 Actions

The metabolic effects of adiponectin are similar to those that happen by the activation of the 5`-AMP-activated kinase in liver and muscles. Therefore; the adiponectin action could be through the stimulation of AMPK (Kola *et al.*, 2006). The main target tissues for adiponectin action are skeletal muscles and liver (Guerre-Millo, 2008). In skeletal muscle adiponectin regulates lipid metabolism via the activation of AMPK. It stimulates the fatty acid oxidation in mice (Fruebis *et al.*, 2001). Adiponectin stimulates the synthesis of protein and inhibit it is degradation. Therefore a hypothesis arose for the role of adiponectin in the skeletal muscle growth (Yamauchi *et al.*, 2001).

In the liver, adiponectin increases fatty acid oxidation and inhibits fatty acid synthesis through the stimulation of AMPK (Yamauchi *et al.*, 2001). Furthermore adiponectin increases insulin sensitivity in hepatocytes, consequently the glucose production decreases (Berg *et al.*, 2001). Low

adiponectin concentrations have been found in association to liver disease. Low serum adiponectin concentrations were found in patients suffering from chronic hepatitis (Durante-Mangoni *et al.*,2006). Another finding of decreased adiponectin concentrations was related with the fatty liver disease in rodents (Neumeier *et al.*, 2006).

1.3.5.7 Functions

Adiponectin has many physiological functions including: actions on glucose and lipid metabolism, producing an insulin sensitizing action (Stefan & Stumvoll, 2002), appetite regulation, an anti-inflammatory action (Weyer *et al.*, 2001), regulating cardiac function, angiogenesis, vascular remoulding, regulation of blood pressure, blood coagulation and immunity (Hill *et al.*, 2009). Adiponectin has been suggested to play a role in the maintaining healthy body weight (Hill *et al.*, 2009).

This catalogue of adiponectin actions became easier to understand with the discovery of family of adiponectin receptors (Yamauchi *et al.*, 2003a). Adiponectin 1 receptors (AdipoR1) are expressed in skeletal muscle and adiponectin 2 receptors (AdipoR2) are expressed in liver (Nishida *et al.*, 2007). In addition T – cadherin receptors on endothelial cells expressed in the cardiovascular system, nervous system and muscles, bind hexametric and HMW adiponectin (Hug *et al.*, 2004). These receptors are also expressed in other tissues such as brain (Yamauchi *et al.*, 2003a), pancreatic cells (Kharroubi *et al.*, 2003), also in the macrophages and atherosclerotic lesions (Chinetti *et al.*, 2004).

AdipoR1 receptors have a high affinity for globular adiponectin and a low affinity for full length adiponectin (Kharroubi *et al.*, 2003). AdipoR1 receptors mediate adenosine mono phosphate kinase (AMPK) activation which increases glucose uptake and fatty acid oxidation in skeletal muscle (Kharroubi *et al.*, 2003). AdipoR2 receptors have an intermediate affinity for both globular and full length adiponectin. This seems to be mainly responsible for mediating the effects of the full length adiponectin in the liver, through the activation of AMPK (Yamauchi *et al.*, 2003a). However;

AdipoR1 and AdipoR2 have not yet been detected in the human plasma (Garaulet *et al.*, 2004). The T-cadherin receptor on vascular endothelial and smooth muscles is known to relate to atherosclerosis (Takeuchi *et al.*, 2007, Tilg & Moschen, 2006).

Adiponectin infusion into the liver increases the effects of insulin. It reduces the expression of gluconeogenic enzymes and suppresses glucose production (Combs *et al.*, 2001).

Adiponectin has an anti-inflammatory action. It enhances the effect of anti inflammatory cytokines like interleukin 1 and interleukin 10 (Wolf *et al*, 2004). Adiponectin also suppresses the production of IL-6 and TNF-α in lipopolysaccharide activated human macrophages (Wulster-Radcliffe *et al.*, 2004). Adiponectin has a protective effect against arteriosclerosis, most likely because it suppresses macrophage production of pro inflammatory cytokines (Wolf *et al.*, 2004; Wulster-Radcliffe *et al.*, 2004). It also inhibits monocyte adhesion to aortic endothelial cells and suppresses the transformation of macrophages to foam cells (Ouchi *et al.*, 2001). Vascular anomalies were found in adiponectin deficient mice. These are the result of increased proliferation of vascular smooth muscle cells in response to external arterial injury (Matsuda *et al.*, 2002). Conversely, adiponectin administration to apolipoprotein-E deficient mice reduced the size of the atherosclerotic lesions (Yamauchi *et al.*, 2003b). More recently, studies in adiponectin deficient mice have revealed new roles for adiponectin: protecting the heart against ischemia reperfusion injury and acting as an endogenous antithrombotic factor (Kato *et al.*, 2006).

1.3.5.8 Clinical importance of adiponectin

A decrease in adiponectin concentrations in plasma is related to many aspects of the metabolic syndrome, like insulin resistance, dislipidemia with low concentrations of high density lipoprotein (HDL) cholesterol (Hulthe *et al.*, 2003). A low concentration of adiponectin in plasma is related to several other conditions. These include increased risk of T2D and CVD (Sattar *et al.*, 2006). In addition, there is evidence to suggest a relation between low

adiponectin concentrations and development of liver fibrosis which might also raise the risk of malignancies (Kelesidis *et al.*, 2006).

In the case of CVD and insulin resistance, patients have low total adiponectin concentrations and the adiponectin is mainly found in low concentration of its HMW form (Lara-Castro *et al.*, 2006). Adiponectin gene mutations have consistently been associated with T2D. These conditions are associated with impaired formation of HMW adiponectin hexamers. This finding suggest that the HMW adiponectin is the active form of the hormone (Waki *et al.*, 2003). Thus, it is clinically important to identify a means of restoring normal adiponectin levels, in particular, the HMW form (Guerre-Millo, 2008). It is interesting to note that even moderate weight loss increases adiponectin concentration in plasma by increasing the concentration of the HMW form (Bobbert *et al.*, 2005).

1.3.5.9 Adiponectin and obesity

Adiponectin is different from the other adipokines in that its concentration is inversely proportional to the fat level in the body (Weyer *et al.*, 2001). Positive correlations have been found between the concentrations of adiponectin and HDL- cholesterol. In contrast, it is negatively correlated with triglycerides, low density lipoprotein (LDL) - cholesterol (Cnop et al. 2003). The expression of the adiponectin gene in adipose tissue and its plasma concentration are decreased in cases of lipodystrophy and HIV-associated lipodystrophy, where there is a redistribution of fat with associated metabolic abnormalities such as insulin resistance (Lihn *et al.*, 2003b)

These papers show that the insulin resistance associated with obesity might be a result of reduction in adiponectin concentrations (Lihn *et al.*, 2005). Yamauchi et al. demonstrated that high insulin and glucose concentrations in mice with lipodystrophy and insulin resistance have been ameliorated with the infusion of recombinant adiponectin or adiponectin combined with leptin. This finding indicates that a decrease in the concentration of some adipokines like adiponectin and leptin might lead to some extent to insulin

resistance in lipodystrophic mice (Yamauchi *et al.*, 2001). Maeda et al. recognized that over nutrition leads to reduced concentrations of adiponectin and this is in turn related to development of insulin resistance and diabetes (Maeda *et al.*, 2002). Other findings by Xu et al. Show that the fat content of the liver is reduced by an infusion of adiponectin. One possible explanation of this is that adiponectin reduces the synthesis of fatty acids and increases fatty acid oxidation in the liver (Xu *et al.*, 2003).

Studies of humans who display low adiponectin concentrations indicate that adiponectin plays a role in the development of insulin resistance (Hotta *et al.*, 2000). Obesity, T2D and coronary heart disease reduce adiponectin concentrations (Arita *et al.*, 1999). Accordingly, the concentration of adiponectin mRNA in adipose tissue from lean people is higher than that in obese ones (Lihn *et al.*, 2004). A close relation exists between adiponectin concentration and different indicators of insulin resistance in subjects of normal weight. Intra-abdominal fat has a stronger effect on determining insulin sensitivity than does subcutaneous fat (Cnop *et al.*, 2003). In contrast, the adipocyte derived hormone concentration is mainly determined by the subcutaneous fat (Havel, 2000). Not only this, but also a low level of adiponectin mRNA has been found in the tissues of a group of people who have first degree relatives who are diabetic (Lihn *et al.*, 2003a). Gene expression of adiponectin is negatively correlated with insulin sensitivity (Lihn *et al.*, 2005). Adiponectin has insulin sensitizing, anti inflammatory and anti atherogenic properties (Hotta *et al.*, 2000; Yamauchi *et al.*, 2003b). The concentration of adiponectin in the interstitial fluid of adipose tissue is more affected by acute hyperinsulinaemia than the concentration of adiponectin in blood. Hyperinsulinaemia reduces interstitial adiponectin concentrations by ~ 40%, while the decline in the plasma concentration is only ~ 15%. Moreover, the loss of adiponectin caused by acute hyperinsulinaemia may be different in lean and obese subjects. This could be regulated primarily at a post-transcription level (Murdolo *et al.*, 2009).

1.3.5.10 The effect of exercise on adiponectin

Extensive studies have been done on the mechanism of the effect of exercise on insulin sensitivity. There is no single cause of T2D. It is unlikely that there will be only one mechanism linking exercise and insulin sensitivity.

As mentioned before, adiponectin concentrations decrease in cases of obesity, T2D and coronary heart disease (Arita *et al.*, 1999). In humans, treatment for insulin resistance with thiazolidinediones has positive effects on adiponectin concentrations (Yang *et al.*, 2002). Furthermore, adiponectin concentrations are enhanced by weight loss, which also improves insulin sensitivity (Yang *et al.*, 2001).

The effect of exercise training on adiponectin concentrations in blood is controversial; some studies conclude that adiponectin concentrations are not affected by chronic exercise. Yatagai et al. found that a programme of exercise training in healthy men, lasting for one and half months, enhanced insulin sensitivity without reducing body weight or increasing plasma adiponectin concentrations (Yatagai *et al.*, 2003). This was in agreement with other studies (Hojbjerre *et al.*, 2007; Hulver *et al.*, 2002; Jurimae *et al.*, 2006; Nassis *et al.*, 2005). Punyadeera et al. found that there was no effect of prolonged, moderate intensity exercise on plasma adiponectin levels in healthy lean males (Punyadeera *et al.*, 2005).

In contrast, other studies have found big increases in adiponectin concentrations after exercise training for two and half months. One study tested young and middle aged healthy females, found increases in adiponectin concentrations was accompanied by a reduction in body mass index in the older group but not in the younger group (Lim *et al.*, 2008). Similar increases in adiponectin concentrations were found after short bouts of exercise by overweight males. This exercise programme did not change their BMI (Kriketos *et al.*, 2004). The effect of exercise on adiponectin concentrations in interstitial fluid was investigated by Hojbjerre et al. and they found that acute exercise increased the adiponectin concentrations in

interstitial fluid of subcutaneous adipose tissue in lean and over weight males (Hojbjerre *et al.*, 2007). Previous data suggests that the effect of the exercise might be similar in males and females. It seems that weight loss is an essential factor to increase adiponectin concentrations.

1.4 Exercise

The conversion of the chemical energy to mechanical energy is the main factor that affects the ability of the human to exercise. This chemical energy is obtained from adenosine tri phosphate (ATP). The intra muscular store of ATP is small and can be consumed very quickly during the contraction. So other sources are available to provide ATP for the contractile muscles such as creatin phosphate (PCr), break down of muscle glycogen to lactate and oxidative metabolism of carbohydrate and lipid. Fat metabolism can provide enough ATP to maintain exercise up to 75% of maximal oxygen consumption ($\dot{V}O_{2\,max}$) depending on the aerobic exercise of the individual. While carbohydrate can provide ATP up to 100% of $\dot{V}O_{2\,max}$ in trained or untrained individuals (Hargreaves & Spriet, 2006)

The aerobic system has the ability to produce ATP at a moderate to high rate for several hours. However there are some limitations. At the onset of the exercise the aerobic exercise takes a limited time to reach the required rate of ATP production. The ATP production is less than the requirement of the muscle during the intense exercise. These limitations are filled with the anaerobic system (Hargreaves & Spriet, 2006).

At low intensity exercise (35% $\dot{V}O_{2\,max}$) a low rate of ATP is used. The aerobic metabolism provides most of the ATP needed and there is little need for the PCr breakdown or lactate production. As the exercise intensity increases (65% $\dot{V}O_{2\,max}$) both aerobic and anaerobic pathways are activated to a bigger extent. A large proportion of the required energy is supplied by fat, and muscle glycogenolysis increases. With an increase in exercise intensity (90% $\dot{V}O_{2\,max}$) the need for ATP production increases above that

which can be provided by aerobic metabolism so the anaerobic pathway continues to supply the energy (Howlett *et al.*, 1998; Romijn *et al.*, 1993).

1.4.1 Maximal oxygen consumption

The lungs and cardiovascular system supply oxygen and remove carbon dioxide from the working muscles. Under steady-state conditions the measurement of oxygen consumption per unit time ($\dot{V}O_2$) and carbon dioxide production per unit time ($\dot{V}CO_2$) by mouth correspond to oxygen utilization and carbon dioxide production in the cell (Milani *et al.*, 2006).

"Cardiovascular fitness reflects the maximal amount of oxygen consumed during each minute of near-maximal exercise. Values for maximal oxygen consumption ($\dot{V}O_{2\,max}$) are generally expressed in millilitres of oxygen per kilogram of body mass per minute (ml/kg/min). $\dot{V}O_{2\,max}$ can be determined by a variety of exercise tests that activate the body's large muscles provided exercise intensity and duration maximize aerobic energy transfer. The usual exercise modes include treadmill running, or walking, bench stepping and stationary cycling" (McArdle *et al.*, 2007).

$\dot{V}O_{2\,max}$ is affected by several factors include: age, gender, genes, mode of exercise and body mass. $\dot{V}O_{2\,max}$ decrease gradually with age. It is about 27% lower at age 55 than at 20 years old (McArdle *et al.*, 2007). It is higher in males than females. This difference between genders is mostly related to body composition and haemoglobin concentrations (McArdle *et al.*, 2007). The effect of the mode of the exercise on the $\dot{V}O_{2\,max}$ is mostly related to the amount of the muscle mass activated (McArdle *et al.*, 2007).

1.4.2 Respiratory exchange ratio (RER)

The RER corresponds to the exchange of the metabolic gases in the tissues and it is relatively dependent on the main energy source used for the cellular metabolism, carbohydrate and fat. Thus it is defined as the ratio of

carbon dioxide production ($\dot{V}CO_2$) to oxygen consumption per unit time ($\dot{V}O_2$) (Astrand *et al.*, 2003).

$$(RER = \dot{V}CO_2 / \dot{V}O_2).$$

The percentage of participation of carbohydrates and fat in energy metabolism depends primarily on two main factors: the type of the exercise and the state of physical training (Astrand *et al.*, 2003).

1. Type of exercise: Carbohydrates and fat contribute equally in the supply of the energy at rest and during light or moderate exercise. As the exercise progresses and its duration extends, the contribution of fat to the energy supply increases. Therefore, the contribution of carbohydrate in prolonged exercise is minor. In contrast, the contribution of carbohydrate in energy supply for the high intensity exercise is greater than the fat contribution (Astrand *et al.*, 2003).

The initial training status of volunteers and the intensity and duration of exercise all affect the substrate metabolism. The most important source of energy in high exercise intensity is glycogen in muscles and liver. In the case of moderate to intense exercise muscles metabolise complete intramuscular triglycerides more effectively. When exercise lasts for more than one hour, the glycogen stored in the muscle is run down, and accordingly the contribution of plasma substrates to total energy increases. Plasma glucose concentration remains constant during moderate exercise since the increase of hepatic glucose production is in proportion to the increased muscle glucose uptake (Eriksson & Lindgarde, 1991). The production of glucose in the liver consists of glycogenolysis and gluconeogenesis from precursors like lactate, pyruvate, glycerol and amino acids. At the beginning of the exercise about 75 % of the glucose production comes from glycogenolysis in the liver. Later, glycogenolysis will reduce, due to the depletion in glycogen stores, and gluconeogenesis will contribute in the glucose production up to 50 % of the total. Never the less, this compensation is not enough to balance all glucose requirements. For that reason, the blood glucose will reduce after 90 minutes of exercise (Felig *et al.*, 1982).

2. Training state: oxygen uptake is essential for fat metabolism. Therefore, the use of fat as a source of energy supply is depending on the work rate that provides maximal oxygen uptake (Costill *et al.*, 1979; Rahkila *et al.*, 1980).

1.5 Exercise and insulin sensitivity

Insulin is a peptide hormone with many important effects on different types of cells (Rosen, 1989; Greenspan & Gardner, 2004). It principally has anabolic effects on hepatocytes, myocytes and adipocytes (Greenspan & Gardner, 2004). Insulin stimulates cell growth and differentiation and promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis and lipid storage, and glycogen and protein breakdown (Saltiel & Kahn, 2001). Thus, hyperglycaemia and hyperlipidaemia occur as a result of insulin resistance. Where as a lack of insulin gives rise to protein wasting, ketoacidosis and if not corrected eventually, death. Insulin is essential to all intermediary metabolic processes and its most important action is to promote glucose homoeostasis (Greenspan & Gardner, 2004; Saltiel & Kahn, 2001).

Insulin resistance is usually defined as a decrease of insulin-mediated glucose clearance in the tissues sensitive to insulin and an increase production of glucose in the liver. Peripheral insulin resistance, or decreased insulin sensitivity, is a low ability to use glucose in target tissues, most importantly skeletal muscle. In adipose tissue the most important characteristic of insulin resistance is an increase in lipid storage (Fritsche *et al.*, 2008).

Insulin resistance is the most important feature of the metabolic syndrome, T2D and CVD (Bloomgarden, 2007). On the other hand, insulin resistance is not always pathological, but it might be found in some normal conditions like pregnancy and puberty (Wallace & Matthews, 2002).

Exercise plays a very vital role in the protection against diseases such as T2D and atherosclerosis (Blair *et al.*, 2001). Evidence has been provided by many

epidemiological studies about the inverse relation between the physical activity and the incidence of the T2D (Eriksson & Lindgarde, 1991; Manson *et al.*, 1991)

1.6 Microdialysis

Microdialysis is a relatively new sampling technique used initially for the collection of neurotransmitters in vivo (Church & Justice, 1987; Ramaiya *et al.*, 1997; Zetterstrom *et al.*, 1984; Ungerstedt & Hallstrom, 1987). It is a well known technique for the collection of water soluble molecules with a low molecular weight (Ao & Stenken, 2006). As a result of its success in the collection of the small molecules, it has been interesting to extend its use to more tissues and bigger molecular weight molecules. Particularly it has been shown that the concentration of adipokines in the plasma might not reflect their concentrations at the site of their secretion (Dostalova *et al.*, 2009; Mohamed-Ali *et al.*, 1997)

The site for secretion of adipokines is adipose tissue. Therefore the study of these hormones at the site of their production has a special interest. Microdialysis technique has been used to study secretion by human adipose tissue. It was developed originally to study brain function (Ungerstedt & Pycock, 1974). Recently, microdialysis techniques have been extended to become an important in the measurement of substances in the extracellular fluid of many tissues such as subcutaneous adipose tissue, liver and muscles (Romijn *et al.*, 1993). In particular, it has been used for measurement of adipokines (Ungerstedt 1991).

1.6.1 Principle of microdialysis

The principle of microdialysis is based on the movement of substances by diffusion down their concentration gradients, through a dialysis membrane which separates two different compartments; the interstitial fluid and the fluid perfusing the catheter (Chaurasia, 1999). The perfusion fluid is transported to the outlet tube and recovered so that it can be analysed figure 1-4.

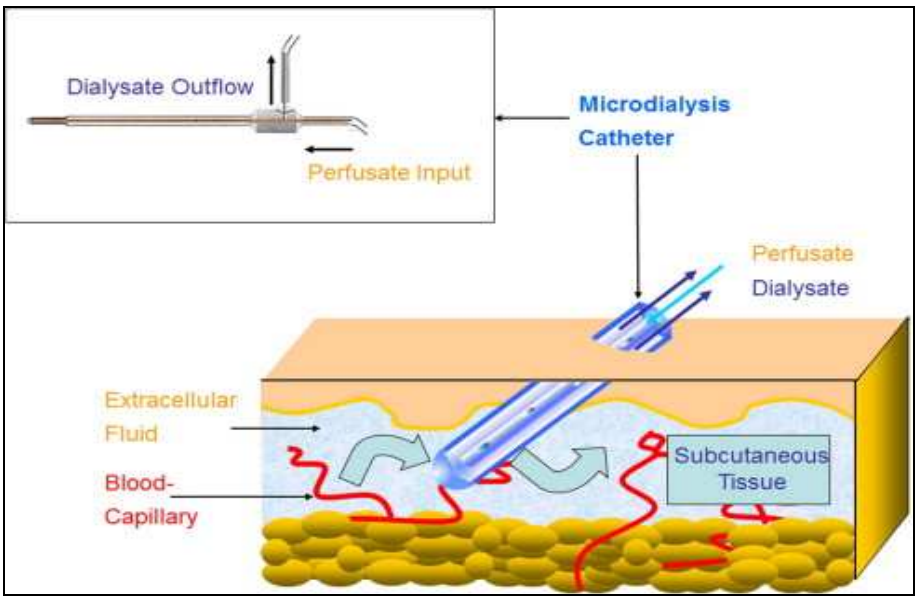


Figure 1-4

Figure shows the principle of the microdialysis (Luppa *et al.*, 2011)

The molecular weight cut off of the microdialysis membranes range between 5 and 100 KD. Therefore the small molecules can pass easily, while the large molecules are rejected or diffuse very slowly through these pores (Ao & Stenken, 2006). To collect samples from particular tissues by using microdialysis, the catheter is inserted into the tissue and a perfusion fluid enters at specific flow rate (0.2 – 5 µl/min). At typical perfusion rates, non equilibrium conditions develope between the substance in the dialysate and its concentration in the extra cellular fluid. The ratio between the fractions recovered to the real concentration is known as relative recovery (Chaurasia, 1999). In contrast, the absolute recovery is the amount of the substance collected from the catheter in specific time. At low perfusion rates less substance is removed and that increases the reliability of the physiological experiment (Wages *et al.*, 1986).

The recovery of adipokines is dependent on many factors including: the properties of the substances, the microdialysis membrane, the perfusate, the tissue (Clausen *et al.*, 2009), perfusion rate and the length of microdialysis tube (Plock & Kloft, 2005). The utilization of a short catheter with fast perfusion rate decreases the diffusion of substances into the dialysate (Clausen *et al.*, 2009).

The microdialysis system contains a pump, catheters or probes and micro-vials. The pump is designed to pump the perfusion fluid at adjustable flow rates ranging from 0.1 to 5 µl/min. It is a battery driven pump which works automatically over periods up to 16 hours. It is easy to handle and portable due to its lightweight. The catheters contain thin dialysis tubes of different lengths, shapes and materials according to the tissue being studied. The semi-permeable membranes typically form the last 30mm at the tip of the concentric catheters or a similar length in the middle portion linear catheters figure 2-4. The perfusion fluid is returned to micro-vials for collection (Tegeder *et al.*, 1999). Each micro vial contains a volume of 200 µl and it takes about 3 hours to be filled at a flow rate of 1 µl/min appendix 6.

1.6.2 Relative recovery (RR)

Relative recovery (RR) means the ratio between the concentrations of the substance in the dialysate to the concentration in periprobe fluid. The relative recovery is depending on many factors (Plock & Kloft, 2005):

1- Diffusion coefficient:

The RR can differ according to the diffusion coefficient (D) of different molecules. The diffusion process of the molecule is directly related to the temperature and inversely to the on the size of the molecule. Consequently it is preferable for the microdialysis experiment to be carried out at a constant body temperature (Kendrick, 1989; Young & Bradford, 1986).

2- Weight cut-off and membrane area:

The substances can pass through the membrane if their molar mass is lower than the cut-off of the membrane (Plock & Kloft, 2005). In addition, the RR is also affected by the length and the area of the membrane, it increases with increase in the length of the membrane (Tossman & Ungerstedt, 1986).

3- Concentration gradient and the composition of perfusate:

The concentration gradient is an essential factor for the diffusion. The concentration changes during the diffusion process so the composition of the perfusate is an important factor in RR (Plock & Kloft, 2005). Osmotic agents are used to improve the RR for macromolecules and to prevent fluid loss in the case of high molecular weight cut-off of up to 100 KDa (Trickler & Miller, 2003).

4- Flow rate:

The flow rate is another important factor affecting the RR. There is an inverse relationship; the best RR recovery can be achieved with a low flow rate. However, this has a negative impact on the volume of the sample and later on the analysis of these samples. It will consequently take a longer time to obtain enough samples (Chaurasia, 1999).

5- Matrix tortuosity

The nature of the sample matrix is another factor affecting the RR (Bungay *et al.*, 1990; Stahle *et al.*, 1991). For the substance to diffuse across the dialysis membrane, it must first diffuse across the surrounding tissue. This tissues contains barriers that could interfere with free diffusion and therefore affect the RR (Chaurasia, 1999)

1.6.3 Performance

Microdialysis can be used to collect samples of the interstitial fluid or deliver samples into this fluid (Plock & Kloft, 2005). Primarily the microdialysis catheters were manufactured to collect small molecules such as neurotransmitters, glucose, glycerol, and lactate (Ungerstedt & Hallstrom, 1987). The molecular weights for glucose, glycerol and lactate lie between 90 to 180 Da. These small hydrophilic substances diffuse freely.

Recently, the recovery of large molecules became possible by microdialysis with the introduction of polycarbonate dialysis membranes (Plock & Kloft,

2005). The cut off of these membranes is about 300 KD reflecting a maximum pore size of about 0.3 μm. This type of microdialysis catheter has been used in human and animal experiments to recover adipokines (Winter *et al.*, 2002), serum proteins, neuropeptides, cytokines and growth factors (Clough, 2005). In addition, microdialysis catheters made from another plastic, polyethersulphone have become commercially available. These have molecular cut offs between 6 to 100 KD. These catheters are well tolerated by tissues and can be used to collect samples from subcutaneous adipose tissue for four days (Clausen *et al.*, 2009).

It is clearly attractive to measure adipokine concentrations in adipose tissues over several days. This could allow direct comparison of concentrations at the same site in response to changes of activity or diet. It is also possible that the performance of catheters may change, either to improve as local trauma after insertion passes off or to reduce if the permeability characteristics are compromised. This longer term performance of the catheters was assessed over 4 days by Clausen et al (Clausen *et al.*, 2009). Changes day by day in concentrations of the adipokines were observed. Adiponectin concentrations were higher on first day after catheter insertion and then reduced by about 33%. In contrast, the concentrations of other adipokines like: IL-1B, IL-8 and TNF- α increased. This could be due to the difference in the roles of these adipokines in local inflammation responses (Clausen *et al.*, 2009). In practice, 1-2 hours was recommended for tissue equilibration to minimise the effect of inflammatory responses on study results (Clausen *et al.*, 2009; Hojbjerre *et al.*, 2007). Rapid changes in the concentration of cytokines and adipokines can occur immediately after the implantation of the microdialysis probe. These changes slow after a few hours.

1.6.4 Advantages and disadvantages of microdialysis

As a method of sampling of adipokines, microdialysis has many advantages. One of the most important features is the possibility of collecting substances at their site of secretion or action (Clausen *et al.*, 2009; Plock & Kloft, 2005). Furthermore, by using microdialysis catheters with an appropriate cut

off, samples can be obtained that do not require time consuming preparation. The samples are ready for a direct measurement (Waga, 2000). The catheters can stay in place for many days and this allows continuous monitoring and collection of sufficient quantities of samples (Buerger *et al.*, 2003). Serial samples can be taken from one subject which aids experimental design and ultimately means that smaller numbers of subjects need to be recruited (Horal *et al.*, 1995).

Nevertheless, there are some negative features associated with microdialysis. There may be tissue trauma at the time of implantation of microdialysis catheter and this could affect local lymphatic filtration for up to six hours (Hildingsson *et al.*, 1996). Even though the insertion technique is modestly invasive, acute tissue responses to the insertion are obvious as local reddening in skin (Ault *et al.*, 1994) . Bacterial infection could result from inadequate sterilisation (Stenken *et al.*, 2010). This will be particularly serious when catheters are inserted into the CNS. However, the use of this technique continues to expand and that suggests that inflammatory problems must be relatively minor. Lastly, the cost of the commercial microdialysis catheters is significant. Each microdialysis catheter currently costs about 100 pounds excluding accessories such as syringes, micro vials and the perfusion fluids. This might matter less in a clinical project but cost is a significant factor in laboratory studies. Catheters with particular permeability characteristics have been made by individual experimenters (Clausen *et al.*, 2009; Hojbjerre *et al.*, 2007; Nielsen *et al.*, 2009). This is beyond the technical competence of almost all researchers and then adds the need for careful sterilisation after manufacture.

1.7 Technique for measurements

1.7.1 ELISA technique

The ability to measure the concentrations of cytokines and other inflammatory mediators with high specificity and sensitivity is very important in this project. Enzyme linked immuno-sorbant assays (ELISA) are

the most common technique used in clinical laboratories and they are the best validated method (Leng *et al.*, 2008)

ELISA techniques have been used in research since 1970s to replace older radioactive isotope labelling techniques which had high risks of contamination (Butler *et al.*, 1978). The ELISA technique relies on comparative antibody/antigen interactions, where the target protein concentration is subsequently determined from a standard curve prepared from a sequence of standards offered with the kit. Two different ELISA techniques are available: sandwich and competitive. The most popular one is sandwich technique. The principle of this method depends on two monoclonal antibodies. One is fixed on the wall of the micro titration well in the first step. The second one is in the conjugate solution labelled with a peroxidase enzyme. It is delivered in the second step. A washing step removes remaining unbound labelled antibodies.

In this case, the complex formed between labelled antibody and the adiponectin is fixed in the wall and reacted with 3,3 ,5,5 – tetramethylbenzidine (TMB). The density of the colour generated is directly related to the concentration of the antigen or adiponectin in the sample. At the end of the incubation 0.5 M sulphuric acid (H_2SO_4) is added to stop the reaction and give the colour of the end point to be read by the spectrophotometer figure (1-5).

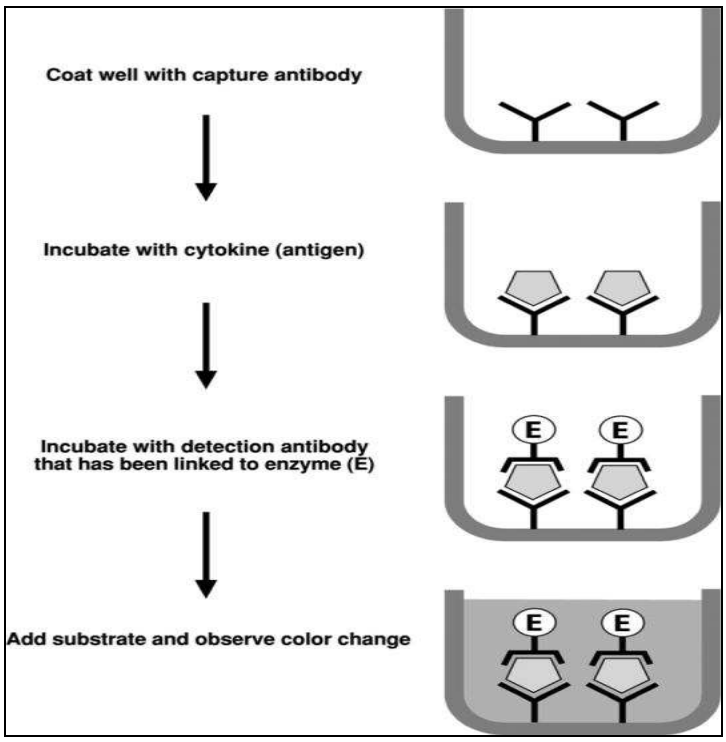


Figure 1-5

ELISA sandwich technique (Leng *et al.*, 2008)

1.8 Conclusion and Aims

It is clear from the literature review above that obesity is a major problem everywhere in the world. It is strongly linked to many serious chronic diseases such as T2D, CVD and insulin resistance. Curiously, whilst women form the majority of most populations there have been fewer studies of these diseases in women than in men even though the prevalence of obesity is higher among females.

Adipose tissues are very vital endocrine tissues. They synthesise many substances such as adipokines and cytokines that play a considerable role in determining insulin sensitivity. It is well known that exercise increases insulin sensitivity. However; it is not clear if this effect of exercise on insulin sensitivity is related to the effect of the exercise on adipokines, particularly adiponectin. Further; most of the studies on the effect of exercise on adipokines were in males. Detection of these adipokines at their site of

secretion has special interest. Microdialysis is a relatively new technique for collecting interstitial fluids which contain substances such as adipokines.

The hypothesis to be tested in this project is that exercise increases adiponectin concentrations. Therefore; the overarching theme of the studies in this thesis was to investigate the effect of acute exercise on adiponectin concentrations in dialysate and plasma samples in healthy young women.

This thesis reports three studies. The aims of the first study were:

- to evaluate the feasibility of using microdialysis catheters
- to investigate any differences in adiponectin concentrations in lean and overweight females.
- to study the effect of a single bout of exercise on adiponectin concentrations.

The second study aimed to compare the adiponectin concentration in plasma samples and dialysate samples collected by 100 KD microdialysis catheters.

The third study aimed to investigate the effect of acute exercise on total adiponectin concentrations and HMW adiponectin concentration in plasma samples.

Chapter 2

General Methods

All experiments and analysis of samples described in this thesis took place in the West Medical Building, School of Life Science, University of Glasgow, between October 2010 to October 2012.

2.1 Ethical review process and approval

All experiments were reviewed and approved by the College Ethics Committee for Non-Clinical Research Projects. It took a long time to gain approval. The initial application was made on 27/1/2010 and the first response from the committee was on 23/2/2010, but the final approval was not given until 17/5/2010. The application forms are shown in Appendix 1. No significant changes were made by the committee. This delayed the start of the experimental work by about four months.

Each volunteer was given an information sheet and the chance to discuss their participation before they agreed to take part. They were asked to sign two copies of a consent form. One was kept by the volunteer and the other kept by the researcher. The information sheets and the consent form are shown in Appendix 2 and Appendix 3.

All volunteers were informed that they could stop the experiment at any time.

2.2 Subjects

Only young healthy women were recruited for the experiments described in chapters 3 and 5. Participants were recruited from students of University of Glasgow. They were recruited by email contact, or via their class moodle site, or posters displayed around the main campus. Lastly, personal contacts were made directly to friends and fellow students.

Information sheets were given to each subject. These included details of the experiment protocol and any related discomfort or risk. The information sheets are shown in Appendix 2. In addition, face to face explanations of technical matters were given. Volunteers were invited to ask questions and told they were free to stop at any point.

Twenty five female volunteers were identified and recruited, but only 15 volunteers completed the first study as described in chapter 3. Their mean age was 22.8 ± 3.0. Their range of ages was between 20 – 30 years. Their anthropometric data is shown in table 3-1 on page 78 of chapter 3. The volunteers were assigned to two groups (lean and overweight) on the basis of their BMI.

The second study recruited six healthy male volunteers. Their mean age was 32.8± 13.1 years (mean ± SD). Their range of ages was between 22 – 58 years.

Lastly for the third study, thirteen female volunteers were recruited for the experiments described in chapter 5. None of these volunteers took part in the experiments described in Chapter 3. Their mean age was 24.3 ± 2.7 years. Their range of ages was between 21-30 years.

2.3 Inclusion and exclusion criteria

The inclusion criteria for participation in the first and third experiment were:

Healthy young women between 20 to 30 years old,
No current prescription medication,
Body mass index between 19 and 29.9 kg/m^2.

The exclusion criteria were:

Illness,
Pregnancy,

First relative diabetic,
Obesity (defined as BMI ≥ 30 kg/m^2,
Participation in vigorous regular exercise.

The inclusion criteria for male volunteers in the second study were: healthy and non obese.

For the last study in this thesis, the volunteers were healthy females, aged between 18 to 30 years. They had a sedentary life style and they had no regular exercise programme. They had normal body weight with a BMI < 25 kg/m^2. The volunteers refrained from exercise during the period of the experiment.

2.4 General plan of the experiment

Volunteers came to the lab for familiarisation. They were shown the equipment and its use was explained. The dates and times for the experiments were arranged.

On the first visit, volunteers signed the consent form and completed the questionnaire shown in appendix 3. Volunteers wore sport clothes. Their weight and height were taken. Their fitness level was assessed as described in section 2.5.6.

Order of the rest and exercise days were randomly arranged. Coin was used for randomisations.

The volunteers were asked to come to the lab in fasted state at 8 am on the days of their tests. They had fasted overnight. The subjects had refrained from vigorous physical exercise at least 24 hours before the experiment. They were allowed and encouraged to drink water during the tests particularly on exercise day.

The details of the catheter insertion are given in section 2.6.3. At the end of recoding on day 1, the pump was disconnected and the sample tube was

covered with a sterile dressing to allow the free movement of the subject. The volunteers were asked to keep the catheter inserted overnight. No infections or redness were recorded. All catheters worked very well next day

Volunteers in the third study followed a similar protocol, but with out catheter insertion. Only blood samples were collected.

2.5 Base line investigations

2.5.1 Weight

Weight for all volunteers was measured using digital balance weighing to 0.1kg. The zero adjustment was set each day. The volunteers wore lightweight clothing and were barefoot. They were weighed with both feet flat on the balance and their arms relaxed in a lateral position.

2.5.2 Height

Their height was measured using fixed stadiometer. The volunteers were barefoot and stood with their back positioned against scale. They stood with their neck and head in a normal position and the arms relaxed in lateral position.

2.5.3 Body mass index

Body mass index (BMI) was calculated as the ratio between weight in kilograms and height in metres, squared.

$$\text{BMI} = \text{Weight (kg)} / \text{Height squared } (m^2)$$

2.5.4 Measurements of the percentage of body fat:

The percentage of body fat was measured by using a Bodystat 1500 Bioelectrical Impedance Analyser (Bodystat, Douglas. Isle of Man). The device is illustrated in figure 2-1. The volunteer removed their shoes and rested on bed for 5 minutes.

Four electrode pads were attached to the hands and feet of the volunteers. These are shown in figure 2-2. The Bodystat 1500 was switched on and details of their gender, age, height, weight and activity level were entered using the three keypads. Once the test had been performed a complete body composition analysis was displayed on the screen within few seconds. This included percentage body fat; lean body mass, total body water, metabolic rates, BMI and impedance are also displayed on the screen.

Figure 2-1 Bodystat 1500

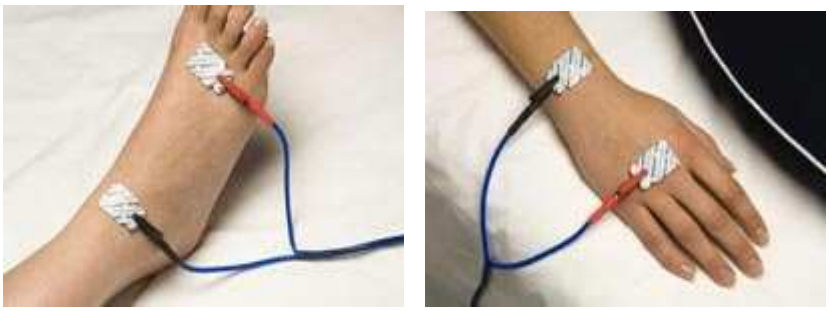


Figure 2-2

Body stat 1500 in use. These photographs show the points of attachment of the electrode pads to the foot and hand of a volunteer.

2.5.5 Measurement of the abdominal skin fold thickness

All measurements of abdominal skin fold thickness were performed by the same researcher using the same Harpenden skin fold calliper (http://www.harpendencalipers.co.uk). The abdominal skin fold was measured for the volunteers while they were standing. The site of

measurement was marked four centimetres from the midpoint of the umbilicus on the right side. The skin was pulled between thumb and index finger of the researcher's right hand. The fold was held by the calliper in perpendicular position for about two seconds. The measurement was done three times and the mean was calculated.

2.5.6 Measurement of maximal oxygen consumption using a sub-maximal test ($\dot{V}O_{2\,max}$)

A sub-maximal $\dot{V}O_{2\,max}$ test was performed by all subjects using standard Douglas bag techniques whilst volunteers walked on a treadmill (Woodway Ergo ES2, Germany).

Each volunteer wore a head set to support a mouth piece and valve. The expired gas was collected in a Douglas bag for periods of 2 minutes. The volunteer's heart rate was measured with a Polar Heart Rate Monitor.

The test started by the volunteer standing at rest for 3 minutes. An expired gas sample was taken during this period. The treadmill was switched on and the speed was increased, starting at 2.7 km/h and rising up to about 8 km/h. The speed was increased in stages lasting three minutes until subject reached 80 to 90% of her maximum heart rate. The precise speed depended on the age and heart rate of the subject. During this process five or six expired gas samples were taken. The maximum heart rate (MHR) was predicted using the equation, MHR = 220-age.

The oxygen and carbon dioxide fraction in the gas samples were measured using a Servomex 1440 gas analyser (Servomex Limited, Crowborough, England). This was calibrated each day using gases of known concentration (Appendix 4). The volume of the gas inside the Douglas bag was measured with a Harvard Dry Gas meter (Harvard Apparatus Ltd, Kent England). The gas temperature was measured concurrently with a digital thermometer and corrections were made for temperature and barometric pressure. These measurements allowed the oxygen consumption to be calculated using the equations listed below and more details are shown in appendix 5.

$$SWVP = (1.1001 \times t) - 4.19$$

SWVP is saturated water vapour pressure in mm Hg at ambient temperature

$$\dot{V}E_{STPD} = \dot{V}E_{ATPS} \times (BP - SWVP)/760 \times 273/(273 + t)$$

BP is barometric pressure in mm Hg

t is ambient temperature in degrees Celsius

ATPS is ambient temperature and pressure, saturated with water vapour.

$$F_IN_2\% = 100 - F_IO_2\% - F_ICO_2\%$$

$$F_EN_2\% = 100 - F_EO_2\% - F_ECO_2\%$$

$F_IN_2\%$ is the percentage of nitrogen in atmospheric air.

$F_EN_2\%$ is the percentage of nitrogen in expired air.

$F_IO_2\%$ is the percentage of oxygen in atmospheric air, assumed to be 20.93%.

$F_ICO_2\%$ is the percentage of carbon dioxide in atmospheric air, assumed to be 0.03%.

$F_EO_2\%$ is the measured percentage of oxygen in expired air.

$F_ECO_2\%$ is the measured percentage of carbon dioxide in expired air.

$$\dot{V}I = \dot{V}E \times F_EN_2\%/F_IN_2\%$$

$\dot{V}I$ is the inspired air volume per minute

$\dot{V}E$ is the measured expired air volume per minute

$$\dot{V}O_2 = \dot{V}I \times F_IO_2\%/100 - \dot{V}E \times F_EO_2\%/100$$

$$\dot{V}CO_2 = \dot{V}E \times F_ECO_2\%/100 - \dot{V}I \times F_ICO_2\%/100$$

$\dot{V}O_2$ Oxygen uptake (l/min)

$\dot{V}CO_2$ Carbon dioxide production (l/min)

$$\dot{V}O_2\,(ml/kg/min) = \dot{V}O_2\,(l/min) / weight\,(kg)$$

$$\dot{V}CO_2\,(ml/kg/min) = \dot{V}CO_2\,(l/min) / weight\,(kg)$$

A graph was plotted of oxygen consumption and heart rate for each volunteer. A specimen is shown in figure 2.3. The volunteers predicted $\dot{V}O_{2\,max}$ was calculated by extrapolation to expected maximum heart rate. The walking speed associated with 50 % $\dot{V}O_{2\,max}$ was estimated and that was used in future exercise periods.

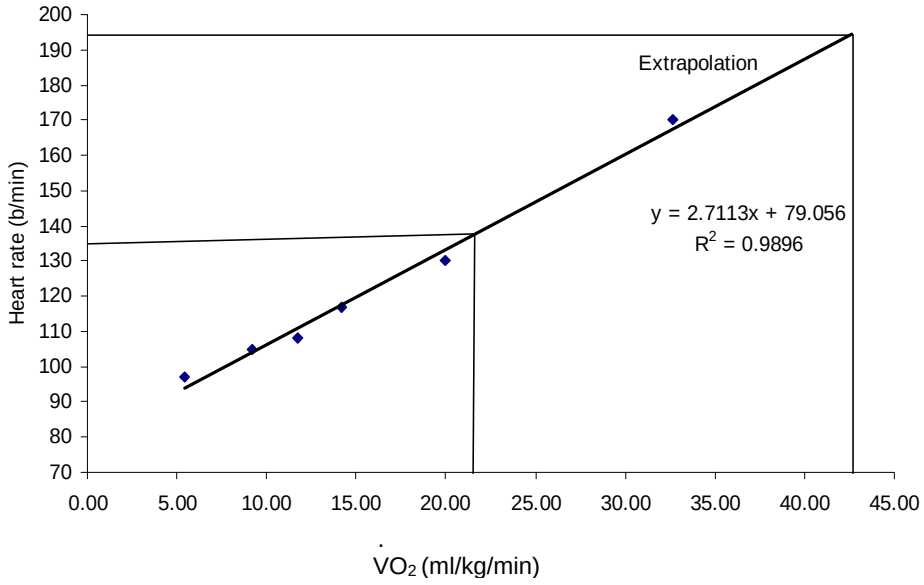


Figure 2-3

A graph showing specimen data for one volunteer. It shows the oxygen consumption in ml/kg/min and the heart rate in beats /minutes during sub maximal exercise. In this case, six samples were taken and a linear trend line was added. This was extrapolated up to predicted maximum heart rate (220 – age). It was a good fit to the data, R^2 = 0.9896. The volunteer exercised up to about 85% of her maximum heart rate. The estimated $\dot{V}O_{2\,max}$ was 42 ml/kg/min and the 50% of $\dot{V}O_{2\,max}$ was 21 ml/kg/min. Therefore the heart rate on her exercise day was around 135 b/min.

For each volunteer the range of walking speeds were 2.5, 4, 5.5, 6.8 & 8 km/hour, with measurement of heart rate and $\dot{V}O_2$. The speed for one hour walk was chosen by picking the speed which delivered the target heart rate. For example this volunteer walks at a speed of about 5.5 km/hr to get a heart rate about 135 b/min.

2.6 Sampling of the interstitial fluid

Microdialysis is a technique used to collect samples of interstitial fluid. It has been used to study fluid from many tissues such as brain, liver, muscles and subcutaneous tissues. All the equipment used was purchased from CMA Company (CMA Microdialysis AB, Solna, Sweden).

2.6.1 Microdialysis catheter

Linear catheters with a 30 mm length of semi permeable membranes were used (CMA 66, Solna, Sweden) appendix 6. The catheter consists of a thin dialysis tube (polyurethane, outer diameter (OD) 0.4 mm connected to semi-permeable membrane (polyarylethersulphone, OD 0.5 mm) at two ends. Details of its introduction are given in section 1.6.1. Briefly, this membrane inserted into the tissue using introducer needle 21 G, which was removed soon after the microdialysis membrane has been inserted into the tissue.

One end of the catheter was connected to an infusion portable pump CMA 107, whose rate can be controlled between 0.5–5 µl/min. The dimensions of this pump are 90 x 50 x 20 mm, with weight of 70 g including battery. This pump is shown in figure 2-6 and Appendix 6-B. In these experiments the flow rate was set to 1 µl/min. Thus was a compromise choice set to deliver a sufficient volume of fluid for analysis and to allow reasonable equilibration with tissue fluids. Fluid returned from the catheter is finally collected in a microvial. The component parts of this system are illustrated below in figure 2-4.

The CMA 66 catheter has a large pore diameter and allows molecules up to 100 KD to diffuse.

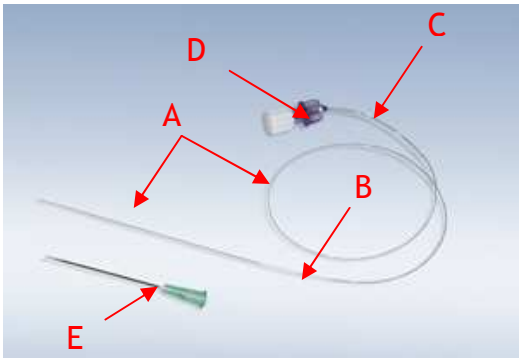


Figure 2-4

CMA 66 high cut off linear catheter. A- 0.4 mm outer diameter dialysis tube. B- 0.5 mm OD semi-permeable membrane. C- 1 mm OD inlet tube. D- Pump connection side. E- 21 G introducer needle 50 mm length.

2.6.2 The perfusion fluid T1

CMA T1 Perfusion Fluid was used. This is a sterile isotonic fluid developed for microdialysis in peripheral tissues. It is packed in glass ampoules of 5 ml. Each ampoule lasted for a single experiment. The T1 fluid contains 147 mmol/l Na^+, 4 mmol/l K^+, 2.3 mol/l Ca^{2+} & 156 mmol/l Cl^- .

The relatively large pore size of the CMA 66 membrane has a problem in that it allows a net loss of perfusate into the interstitial space. The T1 solution balances the crystalloid composition of interstitial fluid but does not balance the colloid osmotic forces. Consequently, Dextran 60 was added at 30 g/l to the perfusate fluid in all experiments (Dextran 60 EP, Pharmacosmos, Denmark). This follows the advice of the catheter manufacturer and Rosdahl et al. (Rosdahl *et al.*, 2000). With this balance achieved, it was always possible to recover fluid from the catheters. In this experiment the volume of the recovered fluid was approximately equal to that expected on the basis of the pumping rate and the time of collection.

The experiments lasted up to 6 hours. The subjects were encouraged to drink at least 100 ml/hour water to maintain normal hydration.

2.6.3 CMA microdialysis catheter insertion

The site of the insertion was marked 4 cm lateral to the umbilicus on the left side. The area of skin around the insertion was cleaned using Alcotip Swabs containing 70% Isopropyl Alcohol. In addition, a local anaesthetic EMLA cream was applied 5 to 10 minutes before insertion, each gram of the cream containing 25 mg of lidocaine and 25 mg of prilocaine (EMLA 5%, AstraZeneca UK Ltd., 600 Capability Green, Luton, UK).

The insertion of the catheter was carried out using an introducer needle. This needle was removed once the membrane was fixed inside the abdominal fat pad. The first insertions used a 21 G needle. This caused problems with damage to the delicate microdialysis membranes. A damaged membrane is shown in figure 2-5. A larger diameter 18 G needle was used for all subsequent experiments. The change was made at the manufacturer's suggestion after rupture of many catheters. The link below shows details of similar insertion procedure (http://www.youtube.com/watch?v=U7ZDhx7tS2A)

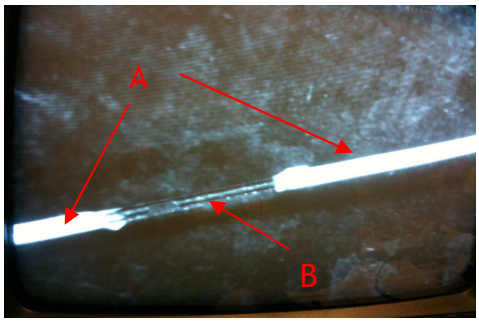


Figure 2-5

Example of broken microdialysis catheter membrane. A- Intact lengths of semi- permeable membrane. B- Exposed inner tube at the site of membrane tear.

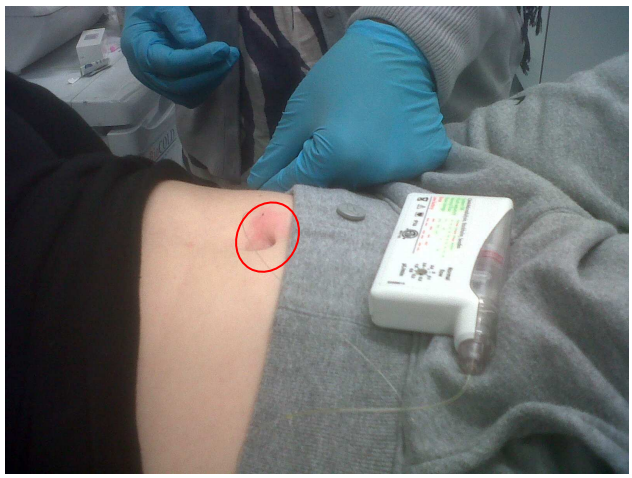


Figure 2-6

Microdialysis catheter insertion. Redness at the site of the insertion disappeared after few minutes.

The insertion sites for the inlet and outlet tubes were covered using Tegaderm film (3M, Health Care, USA). The CMA 107 pump was connected to one side of the catheter with special syringe containing the perfusion fluid. Lastly, the micro vial was connected at the exit end for dialysate collection every 30 to 60 minutes figure 2-6 & 2-7.

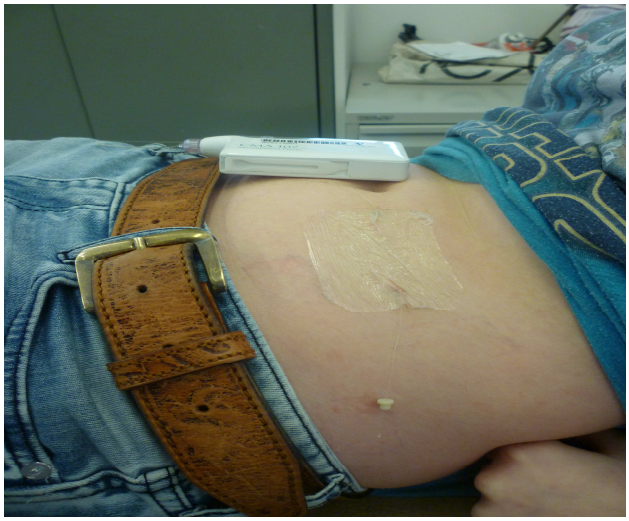


Figure 2-7

This figure shows the microdialysis equipment in use. At the top of the picture, the CMA 107 pump lies on the volunteer's abdomen. In use it is stored in the volunteer's pocket. The pump is connected to the microdialysis catheter by a polycarbonate luerlock-connector and a fine polyurethane inlet tube. The tubing is inserted through the skin using aseptic technique, and the site of insertion is covered with a sterile Tegaderm dressing. The dialysate leaving the catheter is collected in a micro vial seen in the lower part of the picture.

All volunteers finished the experiment. Nobody stopped their participation during the catheter insertion procedure. The insertion and removal of the catheters were done under antiseptic conditions. No catheters were removed during experiment because of infection, soreness or discomfort. Removal of catheters was always clean and no parts were retained. After catheter removal, the sites of inlet and outlet punctures were cleaned, checked and covered with a plaster.

2.7 Blood samples

Venous blood samples were collected from a cannula inserted into a vein in the forearm. 20 G Venflon™ Pro Safety cannulae were used. The needle was removed and replaced by three way valve connecter with a 10 cm connecting tube (BD Connecta™, Sweden). This connection was kept fixed in place via IV peripheral line dressing with transparent membrane (EASI-V, Unomedical, UK). The blood samples were collected in 2 ml tubes containing potassium ethylene di-amine tetra acetic acid (K_3EDTA) (BD Vacutainer systems, Plymouth, UK) for subsequent measurement of adiponectin, insulin, HMW adiponectin, IL-6 and TNF-α. An additional 2 ml of blood was collected in fluoride oxalate tubes (BD Vacutainer systems, Plymouth, UK) for glucose measurements. These anticoagulants were chosen according to the recommendation of the kits. After each collection the cannula was flushed by non-heparinised saline solution (0.9% NaCl, BD PosiFlush™ SP Syringe) to prevent blood clotting and to maintain patency. When single blood samples were required, a butter-fly needle (BD Vacutainer® Safty-lok™, 21G, 0.8 x 19 mm x 178 mm) was used to take blood from an antecubital or forearm vein.

Blood samples destined for adiponectin, insulin and glucose measurement were immediately centrifuged at 1400 g at 4°C for 10 minutes (Universal 320R centrifuge, Germany). Those samples intended for HMWA, IL-6 and TNF-α analysis were centrifuged at 1000 g at 4 °C for 15 minutes (Sorvall, legend RT plus centrifuge, Germany). Plasma was then pipetted into Eppendorf tubes. Each sample was separated into two 0.5 ml Eppendorf tubes and stored at −80 °C up until subsequent analysis. The storage time

was a maximum one month, except for plasma glucose samples which were stored for about six months due to a technical problem.

2.8 Sample analysis

2.8.1 Principle for ELISA technique

All ELISA procedures depend on a sandwich technique, in which two antigenic determinants are directed against two monoclonal antibodies. One was fixed on the wall of the micro titration well in the first step and the other is found in the conjugate solution labelled with a peroxidase enzyme in a second step. The washing process removes remaining unbound labelled antibodies. The complex formed between labelled antibody and the adiponectin fixed in the wall reacts with 3,3,5,5 –tetramethylbenzidine (TMB). The density of the colour directly relates to the concentration of the antigen or adiponectin in the sample. At the end of the incubation 0.5 M sulphuric acid (H_2SO_4) was added to stop the reaction and give the colour of the end point to be read by a spectrophotometer.

2.8.2 Measurement of adiponectin concentrations

The adiponectin concentration in dialysate samples and blood samples was measured using Mercodia Adiponectin ELISA kits (Mercodia AB, Sylveniusgatan, Uppsala, Sweden, 10-1193-01).

2.8.2.1 Preparation of the solutions and dilution of the samples:

The dialysate samples were taken from the freezer and left to thaw at room temperature. This took 20-30 minutes. In addition, the reagent kits were kept at room temperature. All the solutions were prepared as described in the instructions supplied with the kit. Details are given in appendix 7.

Mercodia kits were used to measure the adiponectin concentrations in plasma. The concentrations were typically 3-30 µg/ml. Details are given in section 1.3.5. The adiponectin concentrations in interstitial fluid were much

lower than that in plasma. The dilution factors were reduced to 1:11 to keep the adiponectin within the working range of the kits.

The instructions of the kit provide dilution factor of 101 (1:101) for plasma samples. It was expected that the concentration in dialysate would be less than in plasma. Therefore, this factor was reduced to 1:11 (20 µl of the sample with 200 µl of the sample buffer), for the first two sets of samples.

In subsequent experiments the dilution factor was reduced again to 1:3 (20 µl samples + 40 µl of sample buffer) for the rest of the samples, and 1:2 (30 µl sample + 30 µl sample buffer) for the second study.

However; the dilution factor for the control and the plasma samples in the second and third studies was kept as mentioned in the instructions of the kit. All samples from one individual were constantly run in the same set.

2.8.2.2 Test procedure and concentrations

The procedure followed was exactly as specified in the instructions in each kit Appendix 7. Control samples from the manufactures were tested with each run (Mercodia Obesity Control A and C Human, Sweden). All standards, controls and samples were run in duplicate. After the dilution of the samples and the controls; the plate was labelled to identify the location of every sample.

25 µl of each sample were added into an appropriate well using a variable micropipette (Fimpipette, Thermo Lab System). 100 µl of assay buffer were added to all wells. This plate was incubated for 1 hour on a plate shaker (VELP Sientifica Vortex mixer) at 500 rpm. Thereafter, the plate was washed with a solution provided with the kit six times automatically using a Dynex MRW plate washer. Then, 100 µl of prepared conjugate solution was added to all wells and incubated again on the shaker for another one hour. Next, the washing step was repeated. Later 200 µl of substrate TMB was added to each cell on the plate and incubated at room temperature for fifteen minutes. Finally, 50 µl of stop solution was added. The endpoint was read at

450 nm by a Spectra Max M2 spectrophotometer (Spectra Max M2, California,USA).

Softmax Pro 5.4 software was used to calculate the standard curve to measure the concentration for adiponectin hormone. A specimen curve is shown in figures 2-8.

The results of control samples showed the reliability and consistency of the assays. The inter assay coefficients of variation for obesity controls was 1.7%, while the intra assay coefficient of variation was 2.6%.

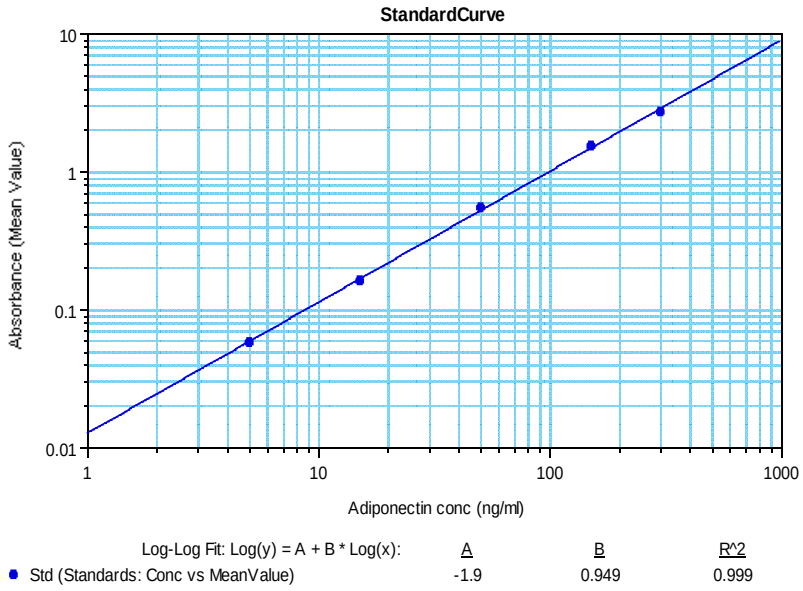


Figure 2-8

This figure shows an example of a standard logarithmic curve used for measurement of adiponectin concentration in the samples. It is obtained from the Spectra Max M2 spectrophotometer and Softmax Pro 5.4 software. Linearity R^2 0.999.

2.8.3 Measurements of insulin concentration

Similar ELISA techniques and Mercodia Insulin ELISA kits (Mercodia reference 10-1113-01) were used to measure the concentration of insulin in plasma samples. Again low and high control samples were tested with each run (Mercodia Diabetes Antigen Control (low & high) Human, 10-1164-01, Sweden)

2.8.3.1 Test procedure and concentrations

The procedure was similar to that described above in section 2.8.2.2. The inter and intra assay coefficients of variation for diabetes control was 2.7% and 4.6% respectively.

2.8.4 Measurement of HMW adiponectin, IL-6 and TNF-α

These hormones were measured using similar ELISA techniques. The analysis kits were bought from R & D Systems (QC01-1, R&D Systems Europe Ltd. Abingdon Science Park, UK). The procedures for the measurement of these three hormones were done according to the manufacturer's instructions. Control samples were tested with each run. Details are given in appendix 8.

2.8.4.1 Concentrations

The concentrations of HMWA, IL-6 and TNF-α were measured using the Spectra Max M2 spectrophotometer and Softmax Pro 5.4 software. The inter assay coefficients of variation for HMWA, IL-6 and TNF-α were 3.5%, 4.7% and 2.5% respectively. The intra assay coefficients of variation were 3.9%, 2.4% and 3.7% respectively.

2.9 Glucose measurement

The concentrations of glucose in the samples were measured using commercially available glucose oxidase reagents supplied by BQ Kits (Valley Centre Dr., San Diego, CA.) appendix 9.

2.9.1 Principle

$$\text{ß-D- Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} H_2O_2 + \text{D-Gluconic Acid}$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{p-HBS} \xrightarrow{\text{Peroxidase}} \text{Immunoquinone} + H_2O$$

Immunoquinone is a red dye, whose concentration is directly proportional to the concentration of the glucose in the sample. The colour intensity was

measured by a Spectra Max M2 spectrophotometer using Softmax Pro 5.4 software.

2.9.2 Procedure

The instructions supplied with the kit were followed as described in appendix 9. Control samples were tested with each run (Horiba ABX, Montpellier Cedex, France). The inter assay coefficient of variation for the controls was 4.8%, while the intra assay coefficient of variation was 3.7%.

2.9.3 Concentrations

The concentrations of all samples were measured manually according the equation:

$$\frac{\text{Sample absorbance}}{\text{Standard absorbance}} \text{ X Concentration of Standard (mg/dl)}$$

To convert the results into SI units (mmol/l), the results were multiplied by ten and divided by 180.

$$\text{mg/dl} \quad \text{X} \quad \frac{10}{180} \quad = \text{mmol/l}$$

2.10 Statistical analysis

Statistical software (Minitab 16) was used for data analysis. Descriptive statistics for all variables were expressed as mean ± SD of the mean. All variables were tested for normality using Ryan- Joiner test.

ANOVAs with a two way repeated measures were applied for normally distributed data. Non parametric statistics were applied for not normally distributed data. Statistical significance was accepted at the 5% level ($P < 0.05$).

Chapter 3

Adiponectin concentrations in dialysate samples

3.1 Introduction

In recent years, more attention has been given to adipose tissue as an endocrine organ, rather than only as a storage site for fat (Kershaw & Flier, 2004; Scherer, 2006). Subcutaneous adipose tissues produce many substances that play a vital role as paracrine/endocrine regulators for many metabolic, inflammatory and immune processes. These substances include adiponectin, leptin and resistin and cytokines such as IL-6 (Kershaw & Flier, 2004).

Low plasma adiponectin concentrations are associated with obesity in humans, diabetes (Weyer *et al.*, 2001) insulin resistance (Hotta *et al.*, 2000) and cardiovascular diseases (Sattar *et al.*, 2006). Goodpaster et al. demonstrated that a 15.1±1.2 kg of weight loss in an exercise program for about 4 months in human subjects improved insulin sensitivity and prevented insulin resistance (Goodpaster *et al.*, 1999).

The effect of exercise on adiponectin concentration in plasma has been studied by many researchers. Conflicting results have been reported in the last 10 years. Some of these differences can be attributed to different exercise protocols. Some studies found no effect of acute moderate intensity exercise on plasma adiponectin concentration (Hojbjerre *et al.*, 2007; Numao *et al.*, 2008; Numao *et al.*, 2011). Others report that even chronic aerobic exercise has no effect on adiponectin concentrations (Boudou *et al.*, 2003; Hara *et al.*, 2005; Hulver *et al.*, 2002; Marcell *et al.*, 2005; Nassis *et al.*, 2005; Yatagai *et al.*, 2003; Punyadeera *et al.*, 2005; Ferguson *et al.*, 2004). On the other hand, a significant increase in adiponectin concentration has been found under the effect of chronic

exercise in some other studies (Bluher *et al.*, 2006; Kriketos *et al.*, 2004; Ring-Dimitriou *et al.*, 2006)

Almost all those studies were on plasma adiponectin concentrations. Only one study was on the effect of the exercise on interstitial adiponectin concentrations in lean and over weight men. They use BMI to decide who was overweight. The samples were collected by 950 KD microdialysis catheters. A Significant increase in adiponectin concentrations was found after one hour of exercise at 55% of maximum oxygen uptake (Hojbjerre *et al.*, 2007). Although obesity is a big problem for both sexes in almost all cultures (Flegal *et al.*, 2010; Katzmarzyk, 2002; Stamatakis *et al.*, 2010) it is surprising that most of these studies used male volunteers.

BMI is not tightly linked to fitness. Problems are known with persons who have unusual characteristics, for example those who are very short in stature or who are very muscular. There are other problems in dealing with women during pregnancy. None of the persons studied in this chapter are of short stature, well muscular or pregnant. Consequently, BMI was chosen following the practice of Hojbjerre 2007.

The original site for secretion of adiponectin is adipose tissue. However, the concentration of adiponectin in adipocytes is lower than the concentration in plasma. It is about 20% lower than the adiponectin concentration in plasma (Hojbjerre *et al.*, 2007). This might be because the dominant form in adipocytes is the trimers and LMW (Bodles *et al.*, 2006) and these probably have a relatively short half-life (Pajvani *et al.*, 2003b). In contrast the major forms found in plasma are the hexamers and HMW (Nakano *et al.*, 1996) and these probably have a longer half-life. The study of these hormones at the site of their production is of special interest. Concentrations in blood may not reflect local concentrations near the site of production. This could be due to dilution by plasma or the action of enzymes or binding to proteins. The concentration of the substance in plasma samples might change over time due to enzyme action or binding. In contrast, micro-dialysate samples do not contain enzymes or plasma proteins since these are too large to pass the membrane. Thus they are ‘cleaner’ samples which do not need any special

preservatives or processing. The microdialysis technique is a sampling method developed about 25 years ago by Ungerstedt. It collects fluids directly from the extracellular spaces and it has been used to study a range of tissues including adipose tissue. It has a variety of advantages and disadvantages. These were reviewed in chapter 1 section 1.6.4.

In a recent paper Clough demonstrated that the use of microdialysis for monitoring of large molecules has been very limited to date mainly due to some methodological challenges (Clough, 2005). In this project this issue revolves round the collection of large molecules through a semi-permeable membrane. Even with these technical problems, the microdialysis technique has been applied to monitor adipokines and cytokines in the extracellular fluid of subcutaneous tissue.

The principle of microdialysis is based on the movement of the substance according to their concentration across the semi-permeable membrane, which separate two different compartments, and this known as passive diffusion. The microdialysis catheter is connected on one side to the CMA pump; perfusion fluid is pumped into the catheter at 1ul/min, and the other end of the catheter is connected to a micro-vial for collection of the sample.

3.2 Aim

This study aimed to investigate:

1- The feasibility of using microdialysis techniques in abdominal fat pads in lean and overweight young women.

2- If the concentration of adiponectin in adipose tissue is different over two days.

3- If the concentration of adiponectin in adipose tissue is different in lean and overweight women.

4- If the concentration of adiponectin in adipose tissue is affected by a short bout of exercise.

3.3 Material and Methods

Volunteers were screened and enrolled for this study as described in chapter 2 section 2.2, following the inclusion and exclusion criteria in chapter 2 section 2.3.

3.3.1 Subjects

The initial contact with potential volunteers was made through personal contact, posters displayed on campus and messages on the University website. The women who responded were all students; except for one member of administrative staff; mostly young and mostly of European origin. Table 3-1 shows the volunteer characteristics. A- Lean group. B- Overweight group.

25 potential volunteers were identified, and 15 subjects completed the whole experiment. The reasons given by the 10 who did not continue were: 2 completed their studies and moved from Glasgow, 3 stopped after the initial anthropometry and fitness tests; 1 had a high resting heart rate, 2 did not meet the inclusion criteria. In addition two volunteers started the catheter insertion phase of the experiments but their catheters did not function correctly. They decided to withdraw from the experiment rather than have a second insertion.

The differentiation of the two groups in this experiment depends on the BMI because it is widely used and this also follows Hojbjerre protocol.

A- Lean Group							
Subjects	**Age (Years)**	**Weight (kg)**	**Height (m)**	**BMI (kg/m^2)**	**Fat (%)**	**Abdominal Skin fold (mm)**	**$\dot{V}O_{2\ max}$ (ml/kg/min)**
Lean 1	21	56.0	1.62	21.3	24.1	21.5	22.0
Lean 2	27	55.1	1.59	21.8	29.8	25.0	36.0
Lean 3	23	48.9	1.59	19.3	35.7	20.0	32.0
Lean 4	24	59.0	1.63	22.2	36.3	21.0	29.0
Lean 5	22	54.7	1.59	21.6	32.9	22.0	38.0
Lean 6	28	67.1	1.68	23.8	30.6	26.0	38.0
Lean 7	20	70.4	1.74	23.3	28.6	19.0	55.0
Lean 8	23	62.0	1.59	24.5	33.2	25.0	35.0
Mean ± SD	**23.5 ± 2.8**	**59.2 ± 7.1**	**1.63 ± 0.05**	**22.2 ± 1.6**	**31.4 ± 4.0**	**22.4 ± 2.6**	**35.6 ± 9.5**

B- Overweight Group							
Subjects	**Age (Years)**	**Weight (kg)**	**Height (m)**	**BMI (kg/m^2)**	**Fat (%)**	**Abdominal Skin fold (mm)**	**$\dot{V}O_{2\ max}$ (ml/kg/min)**
Overweight 1	22.0	67.8	1.64	25.2	35.0	30.0	32.0
Overweight 2	21.0	80.2	1.67	28.8	46.1	41.0	33.0
Overweight 3	22.0	70.6	1.63	26.6	35.0	28.0	35.0
Overweight 4	20.0	77.0	1.61	29.7	43.0	50.0	29.0
Overweight 5	20.0	73.5	1.69	25.7	36.5	29.0	40.0
Overweight 6	29.0	72.2	1.56	29.7	46.4	39.0	24.0
Overweight 7	20.0	67.7	1.54	28.5	43.9	31.0	30.0
Mean ± SD	**22.0 ± 3.2**	**72.7 ± 4.6**	**1.62 ± 0.06**	**27.7 ± 1.9**	**40.8 ± 5.2**	**35.4 ± 8.2**	**31.9 ± 5.0**

Table 3-1

The general characteristics for all volunteers: A- Lean group. B- Overweight group. The highlighted subject in each group was not included in the statistical analysis because of error at the time of sample analysis.

3.3.2 The design of the experiment and sample collection

All volunteers attended the laboratory for three days within a period of two weeks. The first day was given over to information sheets, gaining consent, anthropometric measurements and fitness assessment. Volunteers returned within 1 week for two consecutive days. Subjects arrived at 8 am having fasted overnight, i.e. for at least 10 hours. On the first day the microdialysis catheter was inserted, see Chapter 2 section 2.6.3 for details. After 90 minutes for recovery the volunteer rested for the rest of their visit or

followed a rest, exercise, rest protocol, figure 3-1. The sequence of rest or exercise was randomised. The volunteer returned the next day to complete the other sequence.

The insertion of the microdialysis catheters could damage the tissues at the side of the insertion. To minimise any effect on the results 1.5 hours was allowed from the insertion to the start of the fluid collection. An equilibration period of 1-2 hours was applied in previous studies (Hojbjerre *et al.*, 2007; Simonsen *et al.*, 2008). On the rest day, the subjects rested quietly in the laboratory for about 4 hours. On the exercise day the volunteers rested for 1 hour, exercised for 1 hour at 50% of their $\dot{V}O_{2\ max}$ and rested again for 2 hours.

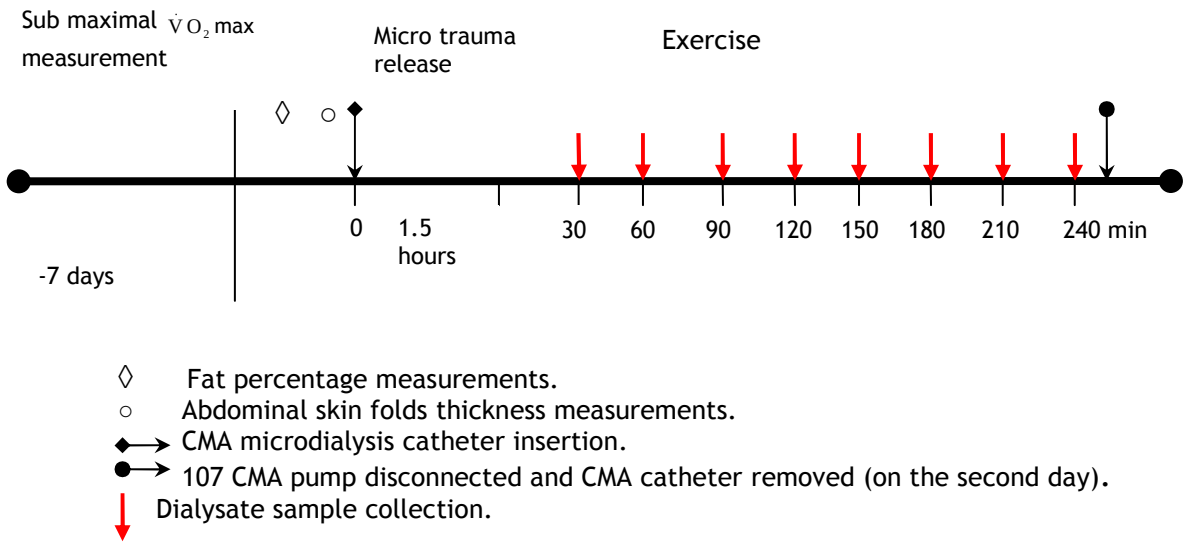


Figure 3-1

Schematic diagram to show the sequence of events during the experiment. In this case the events follow the protocol for the first day, i.e. with catheter insertion and subsequent exercise. In some cases, the volunteers would have rested for the whole session after catheter insertion. On their second experimental day, the first activity is to check that the catheter is still functioning, this took 5-10 minutes, and there was no delay of 1.5 hours to allow for recovery.

3.3.2.1 Sample collection

Dialysate samples were collected in 250 µl micro vials. The catheter was perfused by a microdialysis pump. The flow rate of the pump was kept at 1 µl/min. This flow rate followed the protocol of previous studies. They found

the best relative recovery for adiponectin was at 1 µl/min (Dostalova *et al.*, 2009; Hojbjerre *et al.*, 2007). Thus, in the initial experiments samples were taken every 30 minutes, so the volume in each vial was 30 ± 2 µl. This small volume was troublesome in dilutions and so in later experiments the time of the collection was increased to 45 minutes (giving 45 µl).

3.3.2.2 Sample storage

The samples were kept in plastic bags, in a freezer at -80 ^{o}C up to the time for analysis (New Brunswick Scientific, U725-86). The maximum period of storage was one month. This followed the same protocol as previous studies (Clausen *et al.*, 2009; Dostalova *et al.*, 2009; Hojbjerre *et al.*, 2007; Nielsen *et al.*, 2009).

3.3.2.3 Sample analysis

The dialysate samples were analysed for adiponectin concentration using Mercodia ELISA kits as mentioned before (chapter 2). Each kit has one plate with 96 wells. Samples of two volunteers were tested each run. Each sample was processed in duplicate with a series of standards and controls. Storage of the samples in the freezer allowed the maximum economy and efficient use of reagents in running the tests. Figure 2-8 on page 72 shows a typical standard curve for this analysis. The lowest calibration standard supplied by Mercodia is 5 ng/ml. The adiponectin concentrations of some samples were below this value. These values must be treated with some caution, though the extrapolation appears linear down to detection limit of 1 ng/ml. These low values, below 5 ng/ml were included in the analysis.

3.3.2.4 Statistical analysis

Data for 13 subjects were analysed using statistical software (Minitab 16). Descriptive statistics were calculated for all parameters. Data were presented as mean ± SD. Normality test was applied for all parameters using Ryan Joiner normality tests. Two sample t-tests were used to compare the means of the anthropometric data. Nonparametric statistic was applied for not normally distributed data. Mann-Whitney tests were used to compare the first two samples on the resting day between lean and overweight

groups. Kruskal-Wallis test were used to compare changes across the two groups and to compare changes across the two trials.

3.4 Results

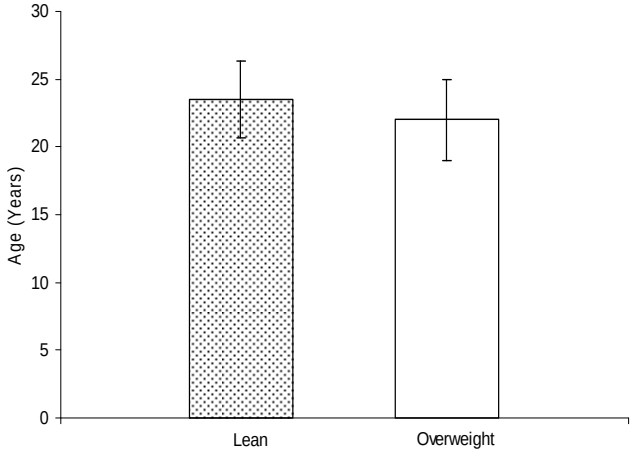
3.4.1 Comparison of two experimental groups

The anthropometric data for the lean and overweight groups is presented in table 3-1. The recruitment to the two groups was not exactly balanced in numbers because of the withdrawals described earlier.

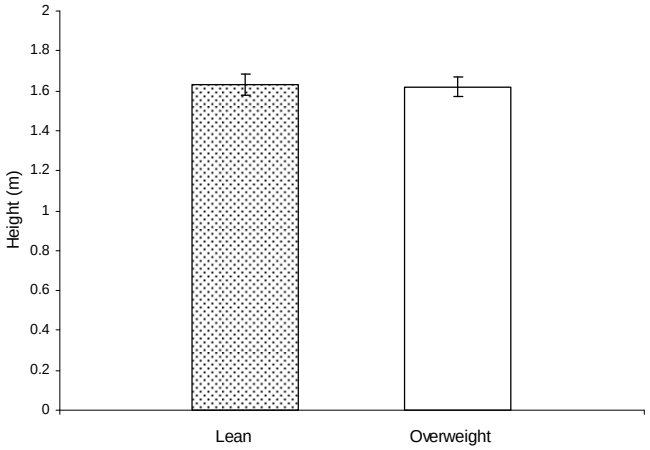
It was difficult to recruit volunteers. After the initial contact was established, some withdrew citing reasons such as: dislike of needles, dislike of exercise tests, and pressure of time. These problems were greater for the overweight group. The introduction of payments to volunteers helped with recruitment and retention.

However, as shown by the data in figure 3-2 there are no significant differences in age or height when tested with a two sample t-test (p values > 0.05). Figures 3-3 and 3-4 show that there were clear differences in body weight, BMI, fat percentage and skin fold thickness (p values <0.05). Interestingly the two groups were similar in fitness as shown by the measurements predicted maximum oxygen consumption (p value = 0.35) figure 3-2 C.

A



B



C

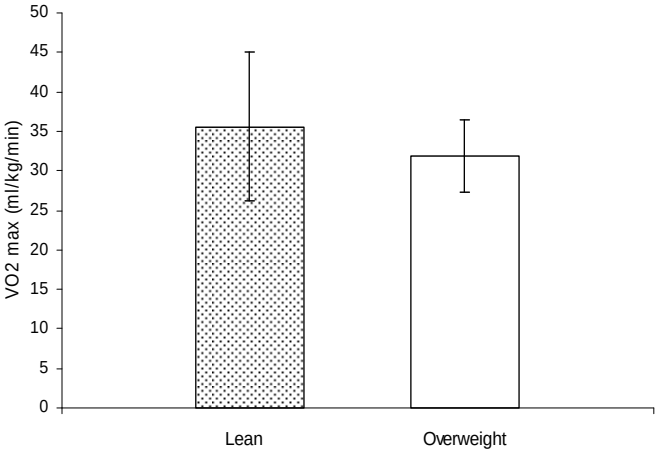


Figure 3-2

These bar charts show the means and standard deviations of the data for -age (A), height (B) and $\dot{V}O_{2\,max}$ (C) for volunteers in the lean and overweight groups. It is easy to see that the two groups are well matched for these characteristics.

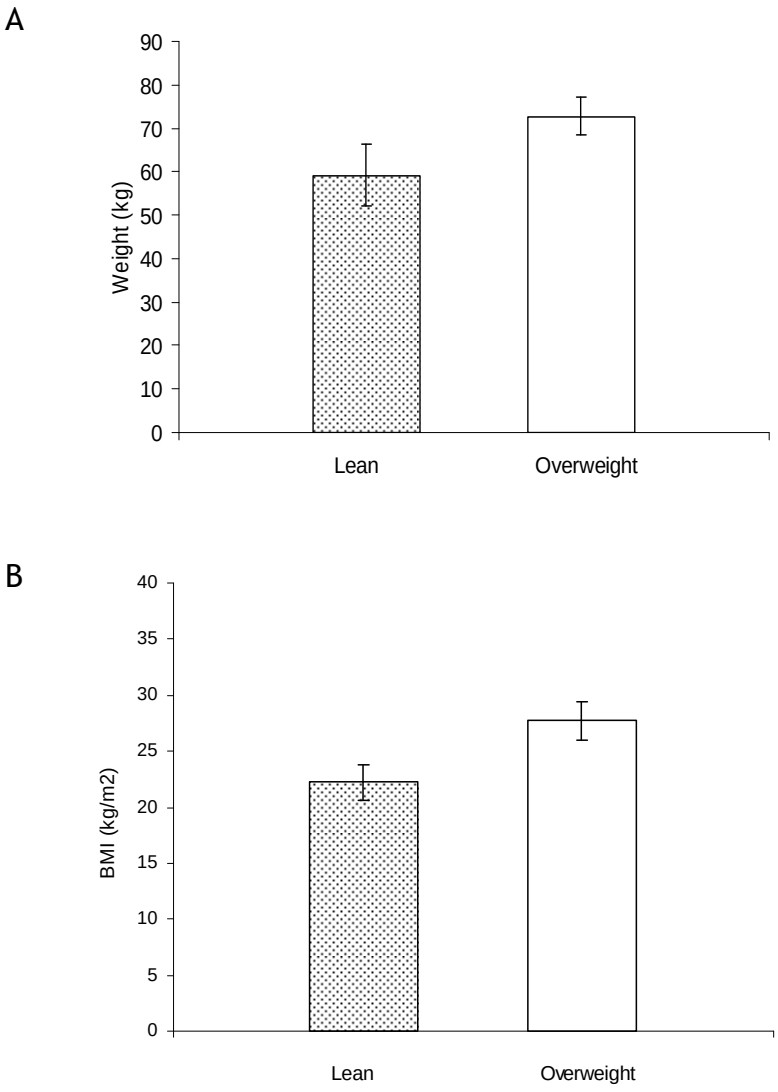
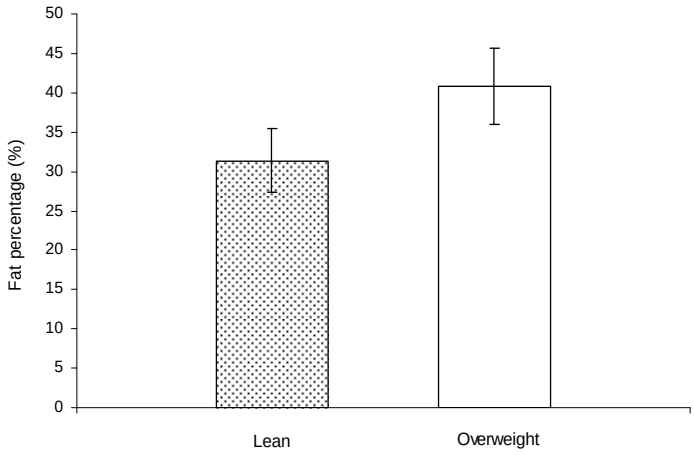


Figure 3-3

These bar charts show the means and standard deviations of the data for body weight (A) and BMI (B) for volunteers in the lean and overweight groups. It is obvious that there are differences in both of these characteristics. These are statistically significant (*P* values < 0.05)

A



B

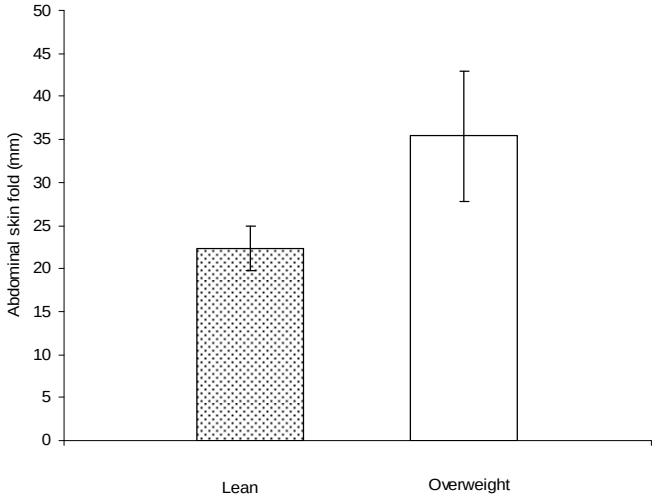


Figure 3-4

These bar charts show the means and standard deviations of the data for fat percentage (A) and abdominal skin fold (B) for volunteers in the lean and overweight groups. It is clear that there are differences in both of these characteristics. These are statistically significant (*P* values <0.05)

3.4.2 Microdialysis catheter functioning

Overall, the microdialysis catheters functioned well over the period of the study. They were well tolerated by the volunteers and after their insertion, they were barely noticed by almost all subjects, although some reddening skin was observed near the entry and exit points figure 2-6 of chapter 2. No

pain or irritation was reported by any subject. No catheters were removed during the experiments because of problems.

In the initial set of experiments an introducing needle was used as recommended by the manufacturer. This caused problems during insertion and of the first eight catheters, four were broken during insertion. The diameter of the introducing needle was nearly the same as the outside diameter of the microdialysis membrane. The insertion process probably rubbed the microdialysis membrane and it broke. An example is shown in figure 2-5 of chapter 2.

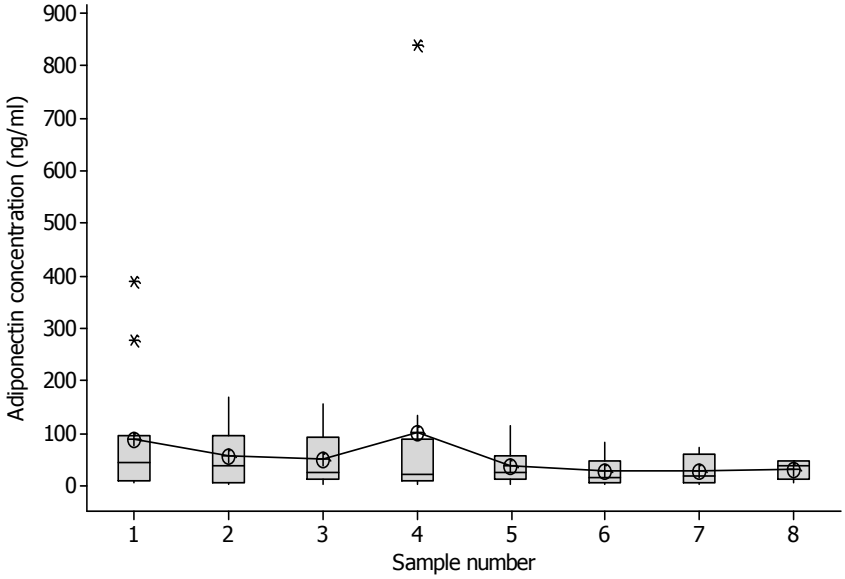
The manufacturers were consulted and they recommended a change from a 21G to a larger bore 18G needle. The link below shows an application of the insertion method using similar CMA catheters and large bore needle http://www.youtube.com/watch?v=U7ZDhx7tS2A. Subsequently, all the insertions with the larger introducing needle were successful and no catheter was broken at the time of insertion.

3.4.2.1 Recovery of the fluid

After insertion, a period of 90 minutes elapsed before samples were taken. During this time perfusion fluid passed through the catheter but the fluid collected was discarded. The recovery of the fluid was successful in all cases. However, the volume of the fluid recovered was very small (30 – 45 µl). This could cause problems with the subsequent analysis. Reading of some samples was below the lower concentration of the standards (5 ng/ml). These samples were included in statistical analysis, which might have a negative effect on the normal distribution of the data. Additionally; several samples had a pale red colour. These samples were very clear but the colour was still obvious. These samples were analysed but the readings were high compared with the uncoloured samples. The three samples which can be identified as statistical outliers are plotted separately in figure 3-5 A. Four other samples attracted attention because of their colour but these were not identified as statistically different from the uncoloured samples. However, these 4 samples were also excluded on the grounds that the colour might affect the optical density of the sample in the ELISA analysis. It is

clear from the figure 3-5A that adiponectin concentrations in the uncoloured samples collected on the first day of the insertion lies in range between 1 and 100 ng/ml. Figure 3-5B shows the box plots of the data re-plotted with the high concentrations from the coloured samples deleted. Figure 3-6 shows the data for the individuals plotted separately. It is easy to see that the adiponectin concentrations were stable in most cases over the period of the collection. In some cases the concentrations fall progressively. In few cases the values rise and fall.

A



B

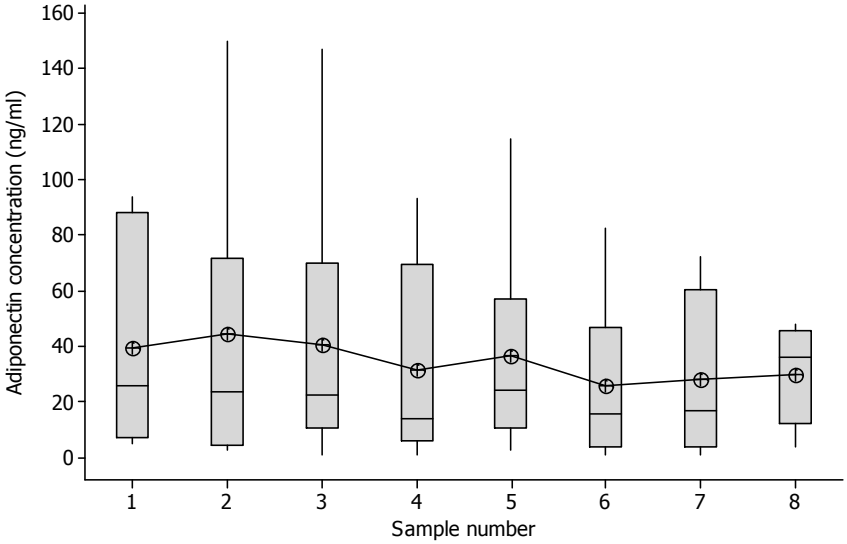


Figure 3-5

The figures show box plots to show the means, medians, quartile ranges and ranges of the adiponectin concentrations in microdialysate samples taken on the day of insertion of the catheter. The upper panel shows all data including the coloured samples described in section 3.4.2.1. The starred values clearly lie outside the expected ranges. The lower panel shows the data re-plotted after removal of the seven coloured samples.

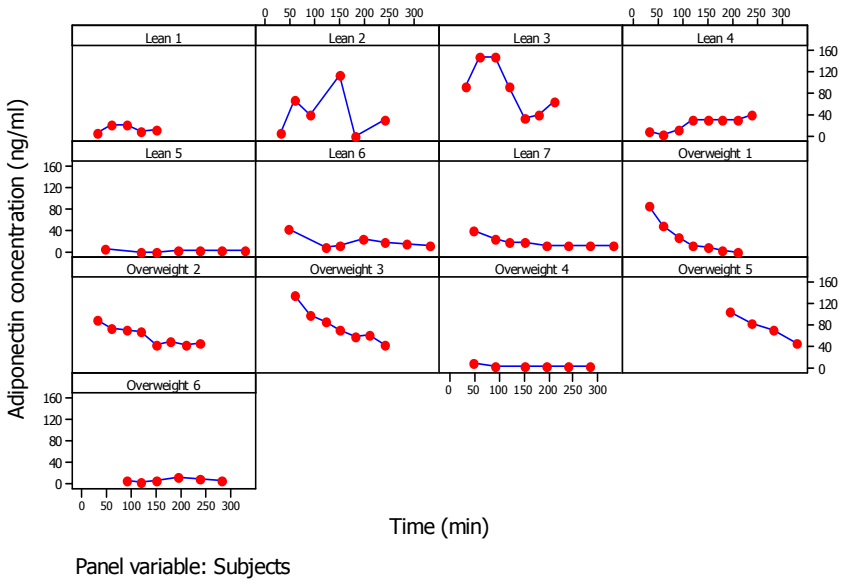


Figure 3-6

The scatter plots show the adiponectin concentrations for each volunteer on their first day of the insertion. Adiponectin concentrations are stable over the period of the collection in many volunteers e.g. lean 5 and overweight 6. Some show a decline e.g. overweight 1and 3. Some show rises and falls e.g. lean 2 and 3. The data plotted omits the very high values identified in 3-5A. Some have missed data either because their value under the detection limit e.g. lean 1 or have high values due to the coloured samples.

3.4.3 Adiponectin concentrations

The distribution of adiponectin concentrations was investigated by conducting a Ryan Joiner test. The samples after deletion of the coloured samples were plotted and are shown below in figure 3-7A. It is clear from the plot and the p value <0.01 that these numbers are not normally distributed.

A

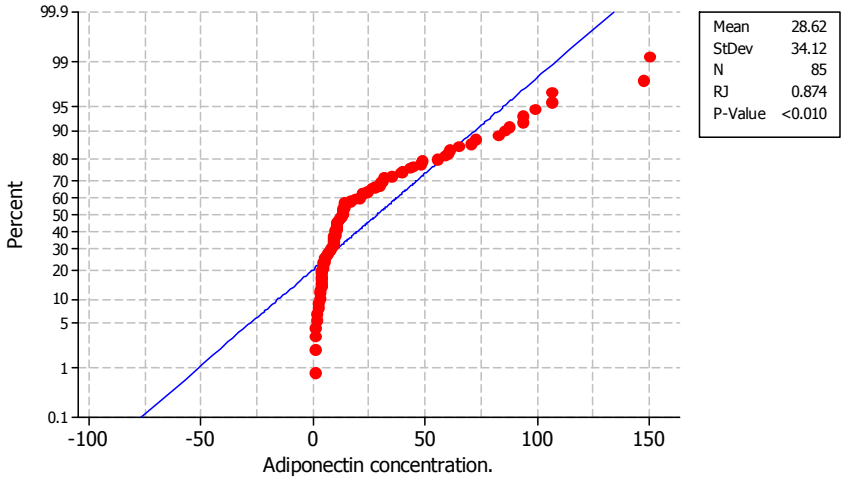


Figure 3-7

Examples of Ryan Joiner plots of the adiponectin concentrations measured on rest day. The figure shows that the data were not normally distributed.

3.4.3.1 Difference between day one and day two of the insertion

One aim of the experiment was to investigate how stable the catheters were over several days. The means of adiponectin concentrations, for the first pair of samples from all volunteers on day 1 and day 2 were calculated. This avoids the effects of exercise later in the collection period. The pairs of means are plotted in figure 3-8. The figure shows that most often there is a lower mean value on the second day. Two volunteers had a higher value on the second day. Interestingly, these volunteers showed low values on the first day. A paired t-test showed that the mean concentration was significantly higher on day 1. The mean values on first and second days were 26.0 ± 15.9 ng/ml and 7.6 ± 5.1 ng/ml (mean ±SD) respectively, *P* value < 0.05.

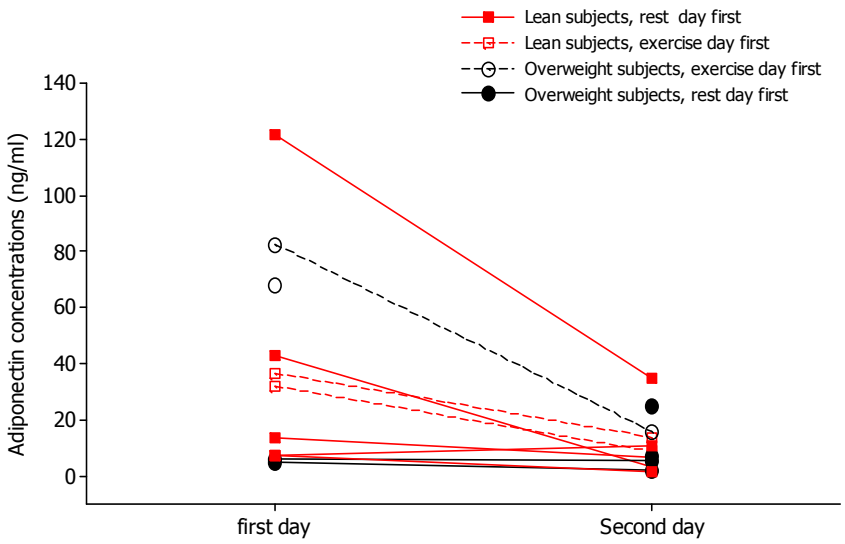


Figure 3-8

The interaction plot shows the mean of adiponectin concentrations of the first two samples for each volunteer on day 1 and day 2. Data for thirteen volunteers is shown. In two cases, only one mean is available. Most of the concentrations were higher on first day than the second day. Two samples show lower concentration of adiponectin on first day.

3.4.3.2 Difference in adiponectin concentrations in lean and overweight volunteers on their rest day

The third aim of this experiment was to investigate the difference in adiponectin concentration between lean and overweight volunteers. As it was mentioned before, rest and exercise days were run in random order for all volunteers. So the rest day could be the first day or the second day of the insertion.

The mean of the first two samples on the rest day were examined for both groups. Nonparametric Mann-Whitney test showed no significant difference. The median values were 13.4 ng/ml and 11.0 ng/ml for lean and overweight groups respectively. The mean values were 30.7 ± 15.9 ng/ml and 23.8 ± 14.9 ng/ml (mean ± SD) for lean and overweight groups respectively, (*P* value > 0.05) figure 3-9.

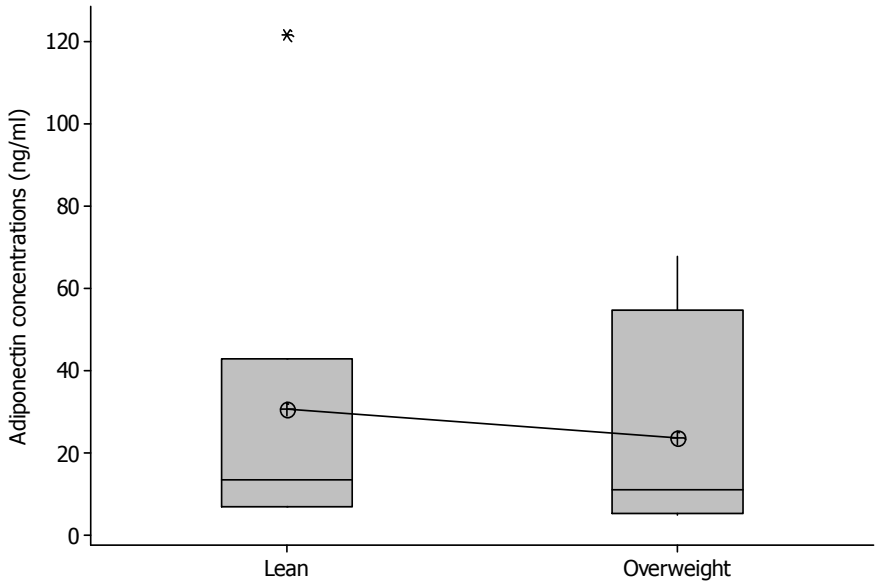
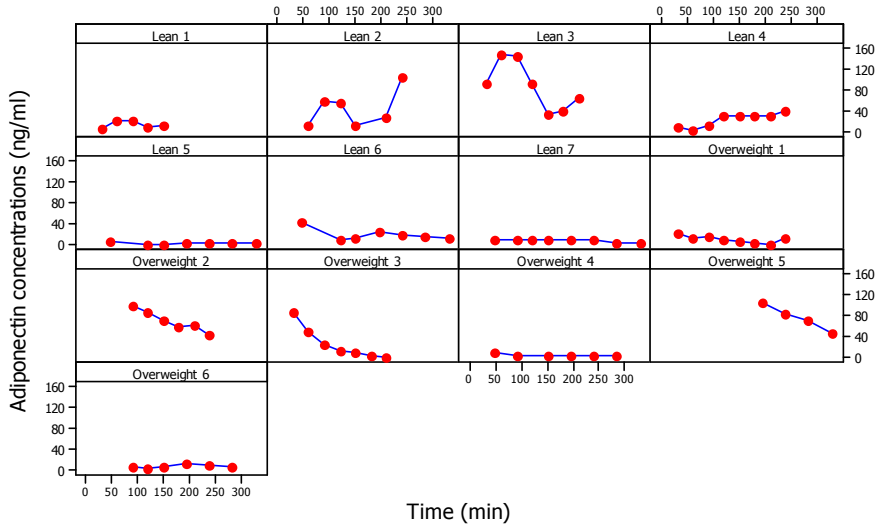


Figure 3-9

Box plots show the mean of adiponectin concentrations of the first two samples for lean and over weight volunteers on their rest day. It is clear from the mean of the first day values; the two groups were not different. (*) is outlier concentration.

This analysis was extended to compare the adiponectin concentrations over the whole period of the rest day i.e. as the fasting continued for another 6 hours. Adiponectin concentrations for each volunteer on their rest day are illustrated in figures 3-10A. There were no significant differences in the mean concentrations in the two groups over the whole fasting period figure 3-10B. The mean values were 28.7 ± 5.1 ng/ml, for lean volunteers and 28.5 ± 5.4 ng/ml (mean ± SD) for overweight volunteers. The differences were tested using nonparametric Kruskal-Wallis test. It showed no significant difference between lean and overweight group (*P* value >0.05). The median was 13.5 ng/ml for lean group and 10.6 ng/ml for overweight group.

A



B

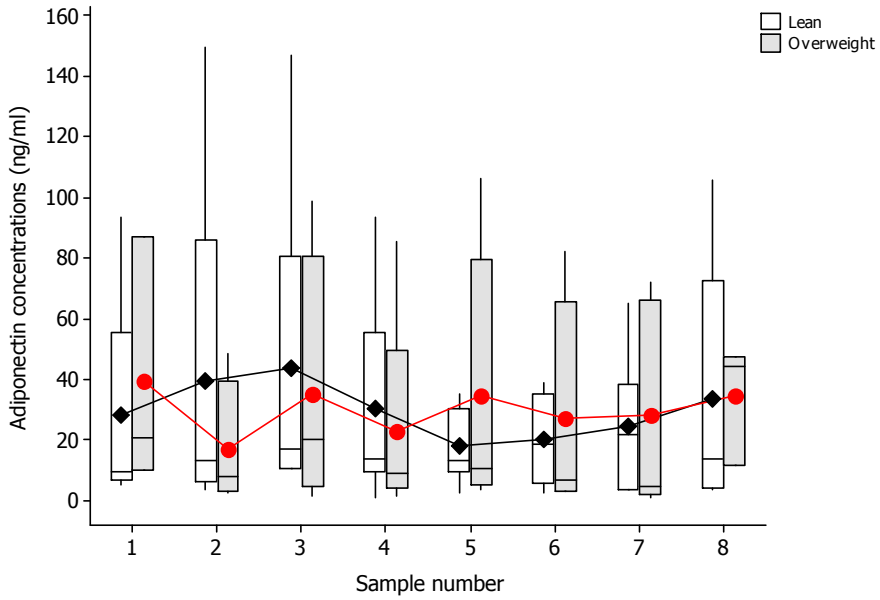


Figure 3-10

A- Scatter plots show the adiponectin concentrations for each volunteer (lean and overweight) over the period of rest. Most of the volunteers show stable adiponectin concentration over time. Two lean volunteers show fluctuations in adiponectin concentrations. Three overweight volunteers show a progressive drop in adiponectin concentrations. B- A box plot shows the adiponectin concentrations for lean and overweight volunteers during the rest day. The mean values for the lean volunteers were more stable but overall the mean values for lean and overweight volunteers were not significantly different. (*P* value >0.05)

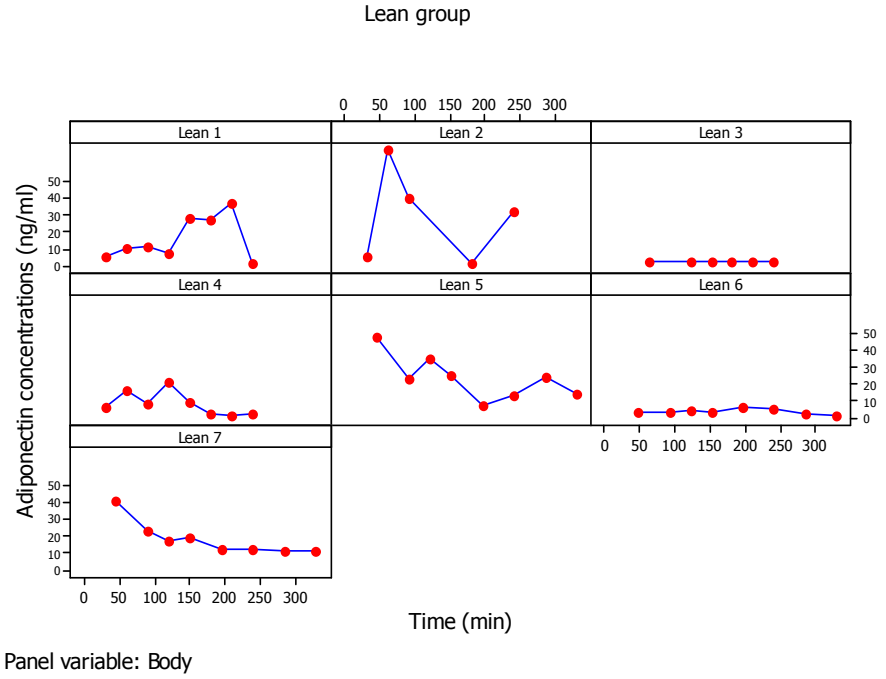
3.4.3.3 The effect of exercise on adiponectin concentrations

The last aim of this series of experiments was investigating the effect of the short bout of exercise at 50% $\dot{V}O_{2\,max}$ on adiponectin concentrations. Both groups exercised by walking for one hour on a treadmill. The randomisation process ensured that this was done of the first day by some volunteers and on the second day by others. The data shown above illustrates clearly that the adiponectin concentrations are stable during periods of rest.

Figure 3-11A shows the adiponectin concentration for each lean volunteer during the exercise day. The period of the exercise started after the second sample. It is clear that there is no obvious effect of the exercise on the adiponectin concentrations. The sampling period for three volunteers lasted for longer time because the collection time for each sample was changed from 30 to 45 min.

Figure 3-11B shows the mean adiponectin concentrations in lean volunteers at points during their exercise day. Visual inspection shows that the mean adiponectin concentrations fall progressively after sample 3, midway through the exercise period. However, these changes were not statistically significant. A non parametric Kruskal-Wallis test showed no significant difference (*p* value >0.05).

A



B

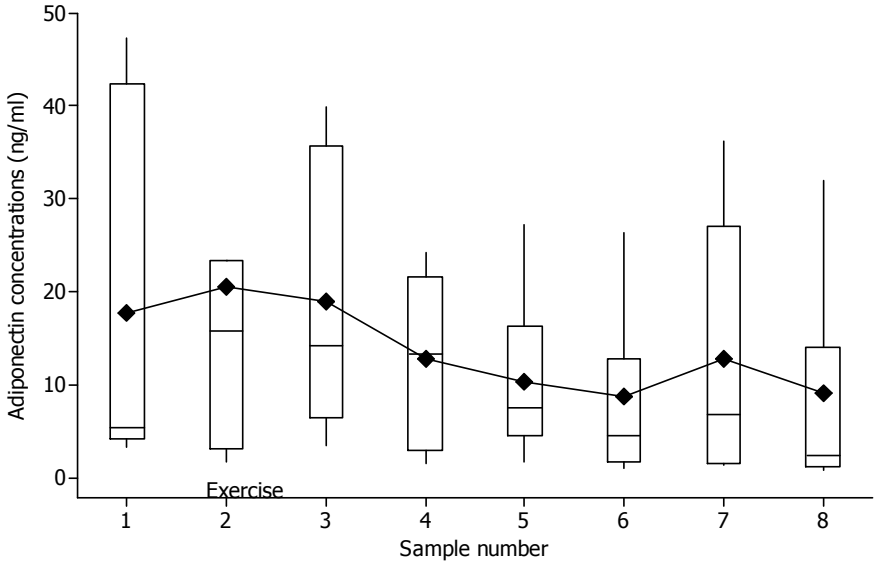


Figure 3-11

A- Scatter plots show the adiponectin concentrations for each volunteer over the period of the exercise. Four volunteers showed fluctuation in adiponectin concentration and three volunteers were stable over the whole period. B-The figure shows box plots to show the means, medians, quartile ranges and ranges of the adiponectin concentrations from lean volunteers on their exercise day. The trends show slight increase after the first half hour of exercise and then a decrease.

Figure 3 -12A shows the data for each volunteer in the lean group on their rest and exercise days. The exercise period started after the second sample. It is clear that almost all volunteers show no change in adiponectin concentrations during or after the exercise. One volunteer, who has the highest adiponectin concentration on rest day, shows a continuing decline.

Figure 3 -12B shows the data for the lean volunteers on their rest and exercise days. Again by eye an apparent difference appears to develop after exercise, but a non parametric Kruskal-Wallis test showed no significant difference (p value >0.05). The median values were 13.5 ng/ml and 11.2 ng/ml for rest and exercise days. The mean values for all samples on the two days were 28.7 ± 5.1 ng/ml and 19.76 ± 1.9 ng/ml on resting and exercise days respectively.

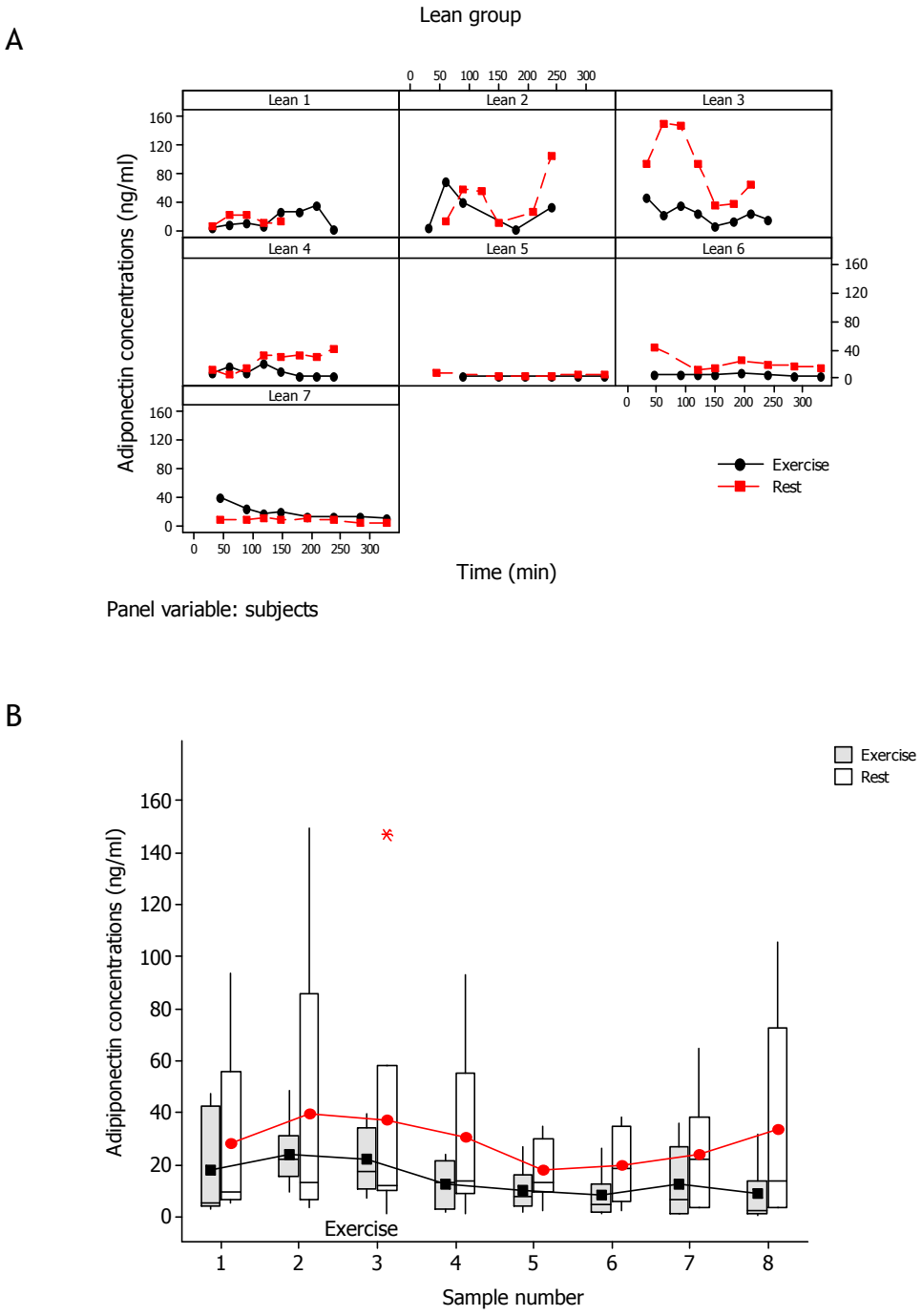


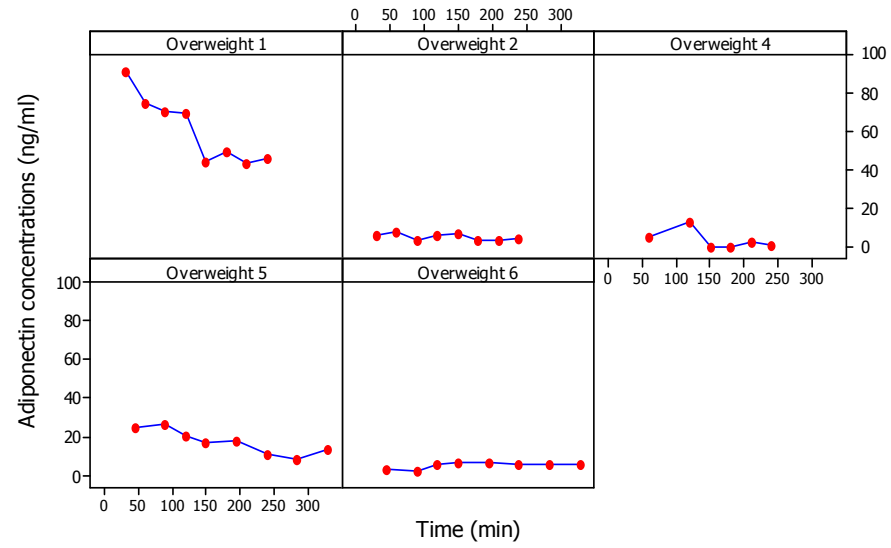
Figure 3-12

A- Scatter plots show the adiponectin concentrations for each volunteer in the lean group on rest and exercise days. B- Box plots show adiponectin concentration in lean subjects on rest and exercise day. Nonparametric Kruskal-Wallis test showed no significant change (*p* value >0.05).

Figure 3-13A shows the adiponectin concentration for five overweight volunteers over the period of the exercise day. The exercise period started after the second sample. It is clear that there is no obvious effect of the exercise on the adiponectin concentrations. One volunteer shows unusually high adiponectin concentration in comparison with the rest of the group. Four volunteers show low and stable adiponectin concentrations.

Figure 3-13B shows the adiponectin concentrations in the overweight volunteers on their exercise day. As with the lean volunteers' data in figure 3-11B, the mean adiponectin concentrations appears to fall progressively after exercise. Again, these changes were not statistically significant. Kruskal-Wallis test showed no significant difference in adiponectin concentration over time (p value >0.05).

A



B

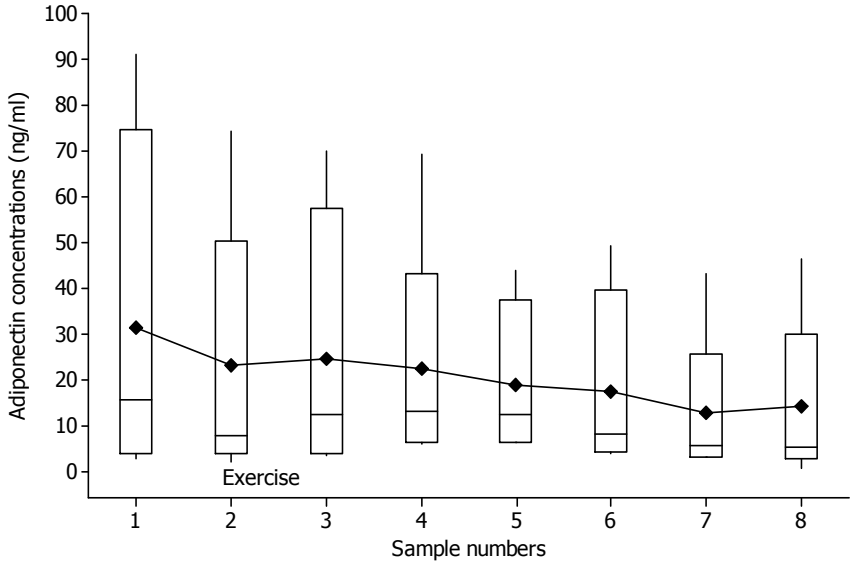


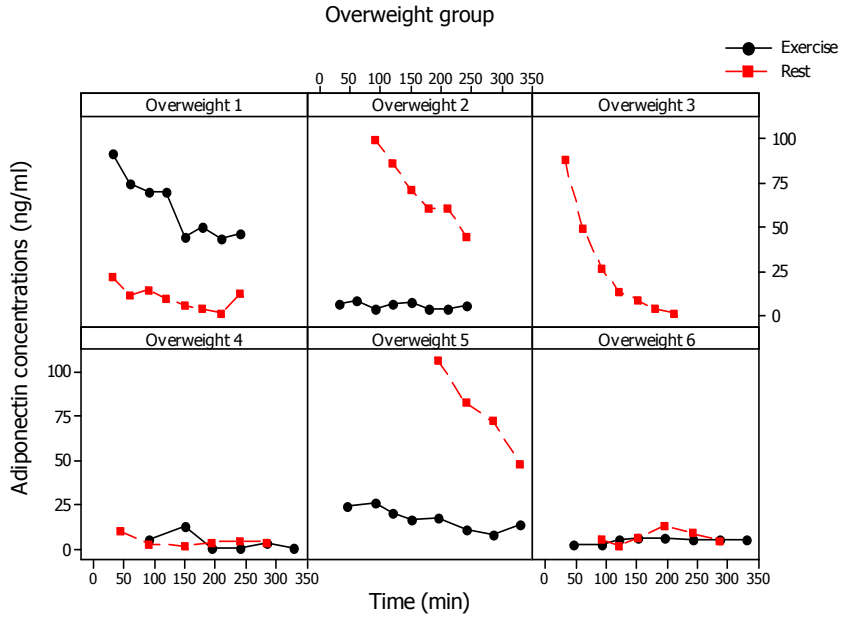
Figure 3-13

A- Scatter plots for five over weight volunteers on their exercise day. Data from the overweight 3 volunteer not available. Almost all volunteers show stable adiponectin concentration. B- The box plots to show the adiponectin concentrations from overweight volunteers on exercise day. Almost all samples were stable all over the time.

Figure 3 -14A shows the data for the each volunteer in the overweight group on their rest and exercise days. The exercise period started after the second sample. It is clear that almost all volunteers show no change in adiponectin concentrations during or after the exercise. The three volunteers, who have the highest adiponectin concentration on rest day, show a continuing decline. One volunteer shows similar pattern on exercise day.

Nonparametric Kruskal-Wallis test showed no significant difference for adiponectin concentrations in overweight volunteers between resting and exercise days (*p* value >0.05) figure 3-14B. The median values were 10.6 ng/ml and 7.4 ng/ml for rest and exercise days. The mean values on the two days were 28.5 ± 5.4 ng/ml and 20.1 ± 4.0 ng/ml on resting and exercise day respectively.

A



B

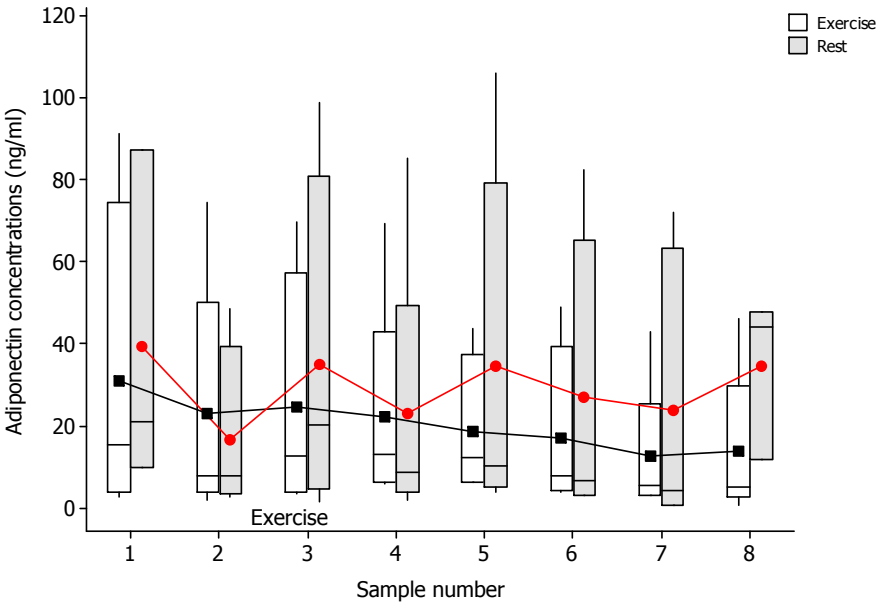


Figure 3-14

A- Scatter plot shows the adiponectin concentrations for each volunteer in the overweight group on rest and exercise days. Data are not available for overweight 3 volunteer on exercise day. B-The figure shows box plots for adiponectin concentration on rest and exercise day for overweight volunteers. It is clear from the figure that there is no significant difference between rest and exercise days for over weight volunteers (*p* value>0.05)

3.5 Discussion

The present study demonstrates the possibility of using microdialysis catheters in SAT in both lean and overweight women. All the insertions were successful with out any infections or problems. It was very successful in terms of fluid recovery, after the initial problems of insertions were resolved. Furthermore, it was an easy technique to use because no processing or preservatives were needed to maintain the samples. All the catheters functioned satisfactorily over the two day period. This was in agreement with one previous study where large pore microdialysis catheters were inserted in SAT of ten men for periods of 4 days. All their catheters functioned fully for two days, 90 % worked on the third day and 30 % on the fourth day (Clausen *et al.*, 2009).

The main difficulty with the microdialysis technique was the small volume of the samples collected. As mentioned in section 3.3.2.1, the volume of each sample collected was 30 µl for the first nine volunteers. This volume was collected because the best recovery of adiponectin was at 1 µl/min (Dostalova *et al.*, 2009; Hojbjerre *et al.*, 2007) and the time of the collection was 30 minutes. The period of collection was increased to 45 minutes. The larger volume collected allowed less dilution of the sample before the ELISA analysis. This improved the ELISA performance, but it also increased the time spent by volunteers in the lab by two hours each day. This did not cause any problems.

The CMA 107 pump had higher available flow rates of 2 and 5 µl/min. These were not used because of the lower recovery rate of adiponectin at those flow rates. Dostalova found that the relative recovery of adiponectin at 2 µl/min was 8.5 ±1.9 %, and at 5 µl/min was 0.7 ± 0.3 % (Dostalova *et al.*, 2009).

This study was an attempt to apply the microdialysis technique to study adiponectin concentrations at the site of its secretion in adipose tissue. The catheter chosen was a commercial product with a pore size of 100 KD. This catheter was chosen because the molecular weight of the adiponectin

hormone as a monomer is 30 KD. The catheter worked successfully in terms of fluid recovery but the concentration of adiponectin in the samples was very low and for some samples below the detection limit of the ELISA kit (section 3.4.2.1). This could be because adiponectin occurs in several forms. The monomer form, 30 KD, is secreted from the adipose tissue. It polymerises to form a low molecular weight trimer of 90 KD (Mangge *et al.*, 2009), medium molecular hexamer at 180 KD (MMW) and an even larger polymer ranging between 240 to 400 KD (Navaneethan *et al.*, 2010). Therefore, 100 KD microdialysis catheters probably collected the monomer but not significant quantities of the larger forms that constitute the greater part of adiponectin in a sample (Hada *et al.*, 2007; Hill *et al.*, 2009; Pajvani *et al.*, 2003b). On the other hand, some samples with a pale red colour had high Adiponectin concentration in the ELISA tests. This was mentioned in section 3.4.2.1 and figure 3-5A. The obvious colour on those samples might be result of entry of haemoglobin molecules into the microdialysis catheter. It is possible that some localised bleeding at the entry site was followed by localised haemolysis leading to a localised concentration of haemoglobin. Its MW is 67 KD and so it is possible that it entered the dialysate. This colour could interfere with the ELISA analysis and explain the high colorimetric reading. It is unlikely for this colour to be due to bursting of the dialysis membrane.

As a result, low concentrations of the monomer form of adiponectin were found. On the other hand; this could confirm the finding that the adiponectin monomer is found only in adipose tissue but not in the peripheral circulation, where only the other forms were detected (Giannessi *et al.*, 2007). Ebinuma et al. demonstrated that the 50% of the total adiponectin are HMW, and 25% are MMW and LMW (Ebinuma *et al.*, 2006). Whereas Beltowski et al. projected that the percentage of HMW were 40%, MMW 35% and LMW 25% of the total adiponectin in human serum (Beltowski *et al.*, 2008).

These finding was in agreement with Dostalova who use the concentric microdialysis catheter with same cut-off (100 KD) to measure adiponectin in subcutaneous adipose tissue. She demonstrated a significant variation in

adiponectin concentration in subcutaneous adipose tissue during a full day study period with low concentration (data not shown) (Dostalova *et al.*, 2009). Clausen et al. used a similar microdialysis catheter (CMA 71) with same cut-off to study SAT. He also found low adiponectin concentrations. This made subsequent Luminex analysis not possible (Clausen *et al.*, 2009).

The data shown in section 3.4.3.1, figure 3-8 shows a significant difference in adiponectin concentrations in microdialysate on the first and second day after insertion. Similar results were found by Clausen et al. (Clausen *et al.*, 2009). They observed a change in adipokines concentrations in the early samples taken immediately after the insertion. This decline continued more slowly during the following day. Both of these sets of observations suggest that the insertion of microdialysis catheter should be done one day prior the actual study starting. This is based on the observations in this study that the catheters were well tolerated by volunteers and that no significant infection problems were encountered.

The lower concentrations of adiponectin on day 2 could have several potential causes. For example, insertion of the catheter could cause local tissue trauma and this could affect the secretion of adiponectin, even though 1.5 hours elapsed between the insertion and the first samples being taken to allow for tissue recovery. This delay was proposed by (Dostalova *et al.*, 2009). A local inflammatory response for the catheter was observed by Murdolo et al. They found that the insertion of the microdialysis catheters increased the concentration of inflammatory cytokines such as IL-6 and IL-8 in lean and obese individuals (Murdolo *et al.*, 2008). An alternative possibility is that psychological stress in the volunteers was greater on the first day.

The data in section 3.4.3.2 shows that the mean adiponectin concentration was not different in lean and overweight volunteers. This might be because the volunteers were moderately overweight, with a mean BMI of 27.7 ± 1.9 kg/m^2, rather than obese. As described in chapter 2, there were problems in recruiting overweight volunteers. The author suspects it might have been even more difficult to recruit obese volunteers. This could have been

worthwhile but additional problems with their health status might have been encountered. Hojbjerre *et.al.*, using microdialysis techniques in male volunteers, also found no significant difference in adiponectin concentration between lean and overweight. In that study the mean BMI of the overweight group was 28 kg/m^2 (Hojbjerre *et al.*, 2007). However, other studies measuring plasma adiponectin concentrations have found a strong negative correlation with BMI in males and females volunteers (Arita *et al.*, 1999; Weyer *et al.*, 2001). This will be discussed in chapter 6.

Lastly, the data from this experiment showed no significant effect of one hour of exercise at 50% $\dot{V}O_{2\,max}$ on adiponectin concentrations which were stable on both resting and exercise days. This might have two possible causes: poor recovery of heavier isoforms of adiponectin or the intensity and duration of exercise might have been too low.

Low concentrations of adiponectin were found in the recovered interstitial fluid (see section 3.4.3.3). These are likely to be principally the monomeric and trimer forms, with MWs of 30 and 90 KD respectively. The cut off of the microdialysis catheter was 100 KD. These might not represent the actual effect of the exercise on total adiponectin. Only one study has been published on the effect of exercise on dialysate adiponectin concentrations. Positive results were found and exercise increased adiponectin concentrations. That study used a particularly leaky type of catheter with a 950 KD pore (Hojbjerre *et al.*, 2007). This catheter is not commercially available.

Using this 950 KD catheter, Hojbjerre et al. found that the concentration of the interstitial adiponectin increases significantly in healthy men after acute bouts of exercise at 55% of their $\dot{V}O_{2\,max}$. The study reported in this thesis exercised women at 50% of $\dot{V}O_{2\,max}$. It is unlikely that the difference between 50% and 55% was responsible for the lack of effect in women volunteers.

The study by Hojbjerre *et al.*(2007) also found that the concentration of interstitial adiponectin in subcutaneous abdominal adipose tissue was 20% of the plasma adiponectin concentration. The same research group have also shown that concentration of adiponectin recovered by the 950 KD catheters can be up to 25 fold higher in plasma than in abdominal subcutaneous tissue (Nielsen *et al.*, 2009).

In conclusion, the commercially available microdialysis system performed well. However, the CMA 66 catheters with a 100 KD cut off used in this study was poor at recovering the larger molecular weight forms of adiponectin. This makes the interpretation of the results difficult especially in case of the effect of the exercise on adiponectin.

Chapter 4

Adiponectin concentrations in plasma and dialysate samples

4.1 Introduction

The data reported in chapter 3 showed that the recovery of adiponectin using 100 KD microdialysis catheters was very low. This confirmed data published by other authors (Dostalova *et al.*, 2009; Clausen *et al.*, 2009). Other authors have shown big differences in adiponectin concentration in plasma and microdialysate recovered from SAT. These studies used the specialised catheters with a 950 KD cut off (Hojbjerre *et al.*, 2007; Nielsen *et al.*, 2009).

Therefore, the purpose of the small study described in this chapter was to compare adiponectin concentrations in the plasma and in dialysate samples. In this study the CMA 66 microdialysis catheter with a 100 KD cut off was used. 100 KD microdialysis catheters were the largest size commercially available

4.2 Aim

The aim of this study was to investigate the differences in adiponectin concentrations in the plasma and in dialysate samples.

4.3 Material and Methods

4.3.1 Subjects

Six male volunteers were recruited for this study from University of Glasgow. It was easier to recruit male volunteers and this avoided retesting females who had already participated in the earlier experiments. This satisfied local ethics committee regulations.

The inclusion criteria were similar to the previous study, the volunteers were healthy males. Consent forms were signed by all volunteers.

The anthropometric data for the volunteers is given below.

No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)
1	29	82	1.72	27.7
2	35	75	1.70	26.0
3	58	98	1.82	29.6
4	28	91	1.88	25.7
5	25	84	1.77	26.8
6	22	64	1.79	19.9
Mean ± SD	32.8 ± 13.1	82.3 ± 11.9	1.78 ± 0.06	25.9 ± 3.3

Table 4-1

The general anthropometric characteristics for the volunteers.

4.3.2 Design of the experiment:

The experimental design was similar except that no exercise was required. Volunteers were invited to attend the lab on two days. They fasted overnight on one day and the other day they ate normally. The sequence was randomised.

On the first day, volunteers arrived at 8 am in the morning and a CMA 66 microdialysis catheter was inserted 4 cm lateral to the umbilicus, on left side. This positioned the catheter in their abdominal subcutaneous tissue. The pump was connected and perfusion started. After one hour collection of microdialysate began, an intravenous cannula was inserted and collection of blood sample began. Five blood samples and five dialysate samples were collected. The dialysate was collected every hour and the blood samples were taken at hourly intervals.

At the end of day 1 the intravenous cannula was removed and the microdialysis catheter covered with a sterile dressing. On the following day the catheter was checked for infections or other problems. The pump was

reconnected and dialysate collection restarted. A new intravenous cannula was inserted. The sample collection protocol was repeated as on the first day.

The microdialysate samples were stored directly at -80^{o}C for subsequent analysis. The blood samples were centrifuged at 3000 rpm for 10 min at 4^{o}C. The plasma was separated into three Eppendorf tubes and stored at -80^{o}C. The maximum storage time in this series of experiment was two weeks.

4.3.3 Samples analysis

Adiponectin concentrations for all samples were measured using the ELISA technique described in Chapter 2.

4.4 Results

4.4.1 Plasma adiponectin concentration

The adiponectin concentrations in first samples of plasma on day one and day two are plotted in figure 4-1. The adiponectin concentrations for these early samples in the five volunteers were within the normal range for healthy adults i.e. 2,000-30,000 ng/ml (Arita *et al.*, 1999). There is no obvious difference in concentration between the two days. The mean values were 5,887 ± 1634 ng/ml and 5,925 ± 1574 ng/ml (mean ± SD) for first and second days, respectively. A paired t-test showed no significant difference between the means (*P* value >0.05). One must interpret these cautiously because of the small numbers.

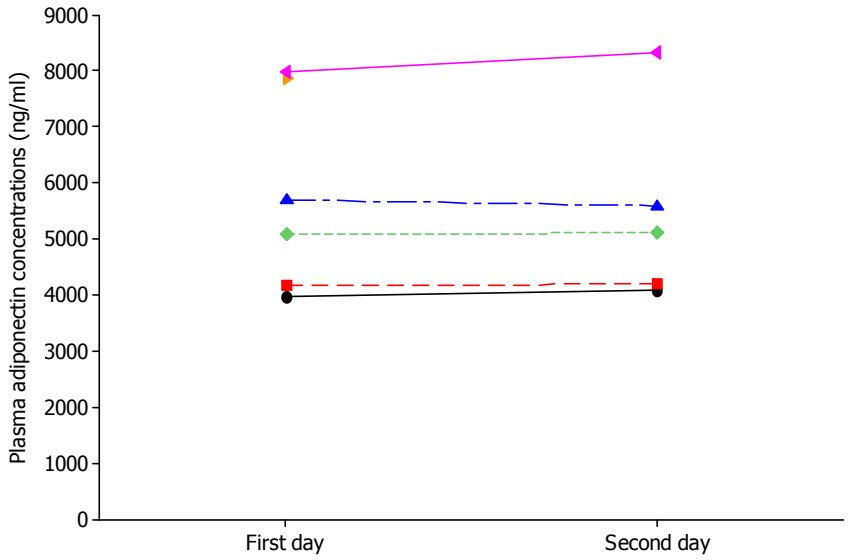


Figure 4-1

Graph shows the first samples of plasma adiponectin concentrations for five volunteers, on first and second day. One volunteer had only one day. It is clear that there is no difference between the first samples on two days for all.

Figure 4-2 A shows the adiponectin concentrations in each of the subsequent samples taken from the volunteers. It appears by visual inspection that the concentrations are very stable over the five hours of sampling. These are re-plotted as box plots in figure 4-2 B.

Given that this was a short series of experiments, there are relatively few data points to include in a statistical analysis. Even though their distribution was not normal (Ryan Joiner $p<0.05$) an ANOVA with repeated measures was conducted. This showed no significant differences with time or trial, fasted, or not fasted.

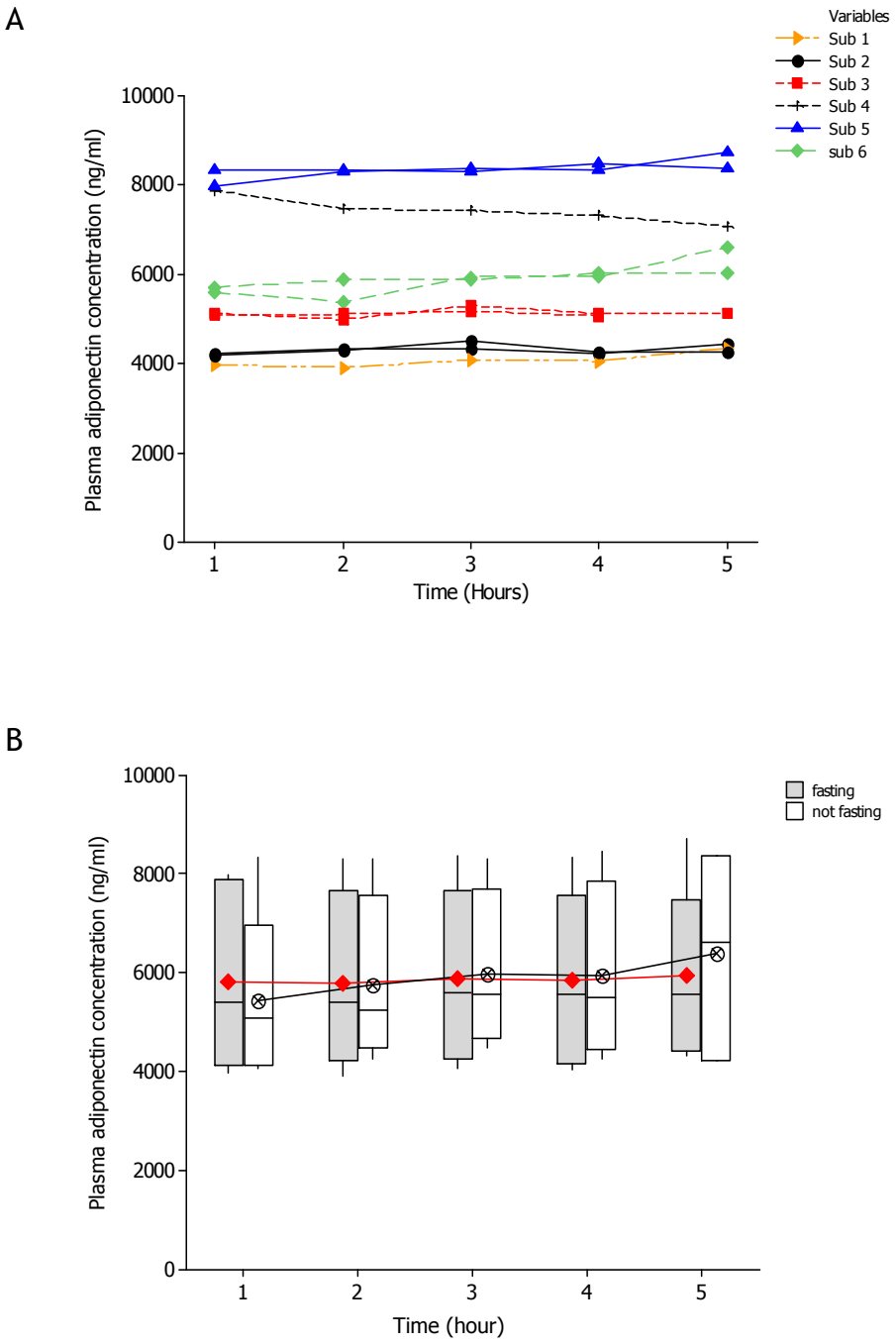


Figure 4-2

A- Scatter plot showing the plasma adiponectin concentrations for all volunteers on two days. Data for each volunteer is plotted separately. B- The box plot shows the mean values for fast and non fasting days. Again it is clear that there is little difference between the two days. Two volunteers did only the first day of the collection. It is clear that there is a small difference between first and second days of the collection.

4.4.2 Dialysate adiponectin concentration

Four of the catheters worked for the whole duration of the experiment. Two volunteers completed only the first day because their catheter stopped working. The samples from one volunteer had adiponectin concentrations below the detection limit of the ELISA kit. Thus, data is available for five subjects on their first day and three on second day of the collection.

Dialysate adiponectin concentrations were similar to that in previous studies reported in chapter 3. The graph below shows data for dialysate adiponectin concentration in three volunteers on their first and second day. Two volunteers showed no difference in adiponectin concentration. One volunteer showed a big decrease on second day.

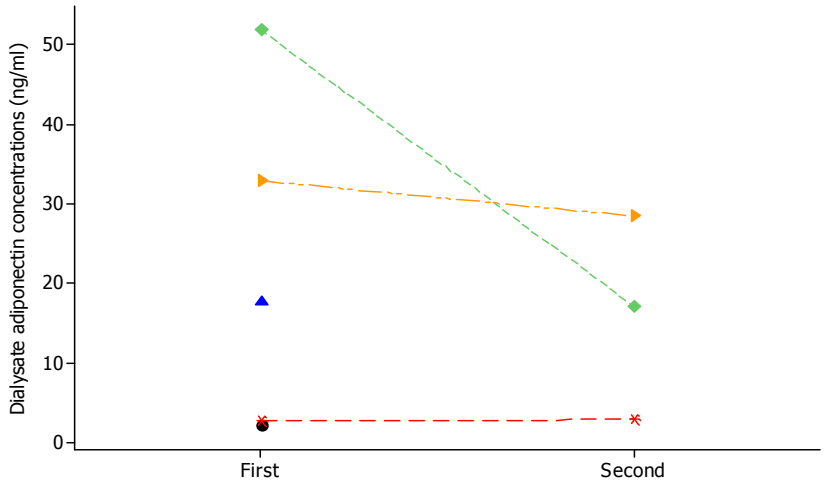


Figure 4-3

Graph shows the first samples of dialysate adiponectin concentrations for five volunteers, on their first and second days. Data for two volunteers are not available for day two. No obvious pattern for the samples. One had a sharp decrease and two had similar concentrations on both days.

Figure 4-4 shows the adiponectin concentrations in each of the subsequent samples taken from the volunteers. It shows one volunteer with higher adiponectin concentrations on first day. His data is very similar to the other

volunteers on the second day. The other volunteers show stable adiponectin concentrations.

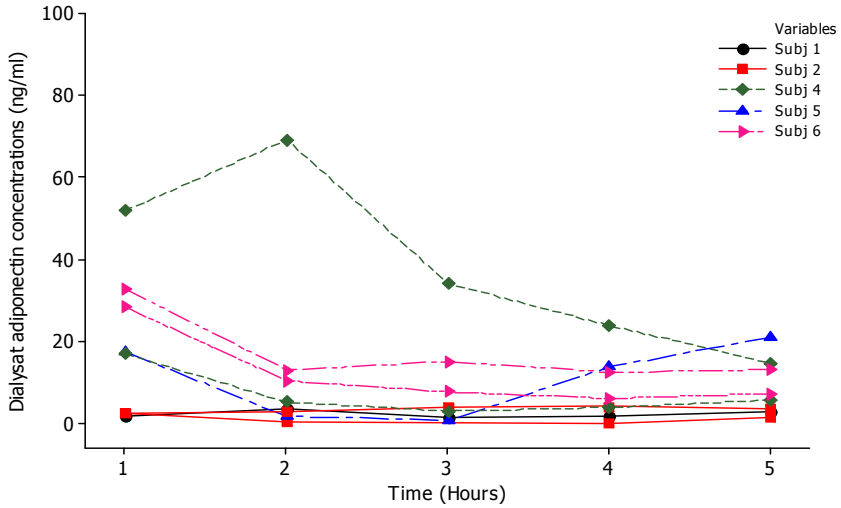


Figure 4-4

Scatter plot showing the raw data for adiponectin concentrations in dialysate for three volunteers on two days. Each volunteer has data plotted for two days by same symbols and same connect line. Two subjects completed only one day.

The data were re plotted as box plots in figure 4.5. The high adiponectin concentrations for the fourth subject on day one were omitted. It is clear from the box plot that the adiponectin concentrations are similar on both days. No further statistical analysis was attempted because of the small volume of data.

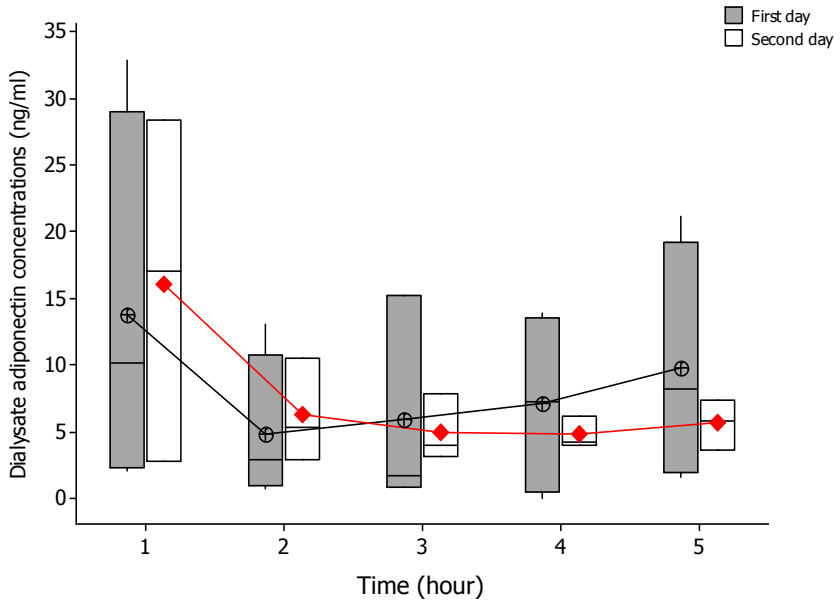


Figure 4-5

Box plot for adiponectin concentrations on first and second days.

Table 4-2 contains the mean adiponectin concentrations for plasma and dialysate samples in all volunteers. It is obvious that there is a very substantial difference between plasma and dialysate adiponectin. The differences are so large that no formal statistical analysis was attempted.

Volunteers	Mean plasma adiponectin concentrations (ng/ml)	Mean dialysate adiponectin concentrations (ng/ml)	Ratio of Plasma/Dialysate Concentrations
1	4075.6	2.5	1630
2	4299.8	2.4	1791
3	5120.3	Below detection limit	---
4	5900.6	23.0	256
5	7434.9	11.1	669
6	8358.9	14.7	568

Table 4-2

Table shows the substantial difference between mean adiponectin concentrations in plasma and dialysate for all volunteers.

4.5 Discussion

The main aim of this study was to compare the adiponectin concentrations in the dialysate collected by 100 KD microdialysis catheters and the plasma concentration.

The adiponectin concentrations in dialysate samples were very low, with mean concentrations between 2.5 and 23 ng/ml. This range is similar to those in Chapter 3 in female volunteers where the means ranged from 3 – 88.9 ng/ml. They are also similar to values of about 2 – 25 ng/ml reported by Dostalova *et al.*(2009).

Table 4.2 compares the concentrations of adiponectin in plasma and dialysate. The adiponectin concentrations in plasma were between 256 and 1791 times higher than those in dialysate. Both sets of samples were tested using the same Mercodia ELISA kits. Indeed the samples were processed at the same time. Thus the difference cannot be due to sensitivity of analysis or degradation of samples in storage. The most likely explanation lies in the dialysate samples containing only the monomeric form of adiponectin whilst the plasma samples contain a range of forms. It was discussed earlier that the microdialysis catheter recovers only a very small fraction of the hormone. That was discussed earlier in chapter 3 section 3-5

Some other studies on males using microdialysis catheters with cut offs at 950 KD demonstrated that the adiponectin concentrations in dialysate samples were up to 25 times lower than the adiponectin concentrations in plasma. This reflects the better recovery of heavier molecular weight forms of adiponectin. The plasma concentrations were similar in this study and in the previously published work (Clausen *et al.*, 2009; Hojbjerre *et al.*, 2007; Nielsen *et al.*, 2009).

It is hard to make direct comparisons of body composition in males and in females. It is worth noting that even at similar BMIs the females almost certainly have a higher percentage body fat (El Ati *et al.*, 2012; Flegal *et al.*, 2010; Kelly *et al.*, 2008). In male adults, adipose tissue constitutes

about 8 to 18% of body weight, while for females it is about 14% to 28%. This percentage greatly increases in case of obesity and could reach 60% to 70% of body weight (Cinti, 2007).

In these experiments, like in chapter 3, the insertion of the catheters was successful. They were well tolerated and no infections or problems were found. The data collected over two days, by microdialysis and plasma samples, allowed the adiponectin concentrations in fasted and non fasted states to be compared. Whilst the data sets are small it appears that fasting has little effect on the adiponectin concentrations.

Chapter 5

The effect of short bouts of exercise on HMW adiponectin concentrations in plasma

5.1 Introduction

On the basis of the data in chapter 3 and 4 it can be concluded that the CMA 66 (100 KD) microdialysis catheters only recover a small fraction of the total adiponectin and this does not reflect the concentrations of all forms of adiponectin. It was very obvious that there was a big difference between the total adiponectin concentration in plasma and the concentration of the monomer or the homotrimer in the interstitial fluid recovered via the microdialysis catheter (Table 4-2). Further, many studies have been done on the effect of exercise on adiponectin concentration in plasma, but very few studies on the effect of the acute exercise on the active form of the hormone (HMWA). In addition almost all of them studied male subjects.

Adiponectin is a complex hormone which occurs in many forms. The active form of the hormone is the heavy molecular weight adiponectin (HMWA) (Hada et al., 2007). HMWA exhibits the greatest binding activity to membrane fractions and the most effective part of the hormone in the activation of adenosine monophosphate (AMP)- activated protein kinase (Hada et al., 2007). Many studies have reported that the HMWA concentration is a better predictor than total adiponectin concentration of many conditions such as insulin resistance, metabolic syndrome and coronary artery disease (Hara et al., 2006; Lara-Castro et al., 2006; Von et al., 2008). In addition, it has very important role in the metabolism of lipid and glucose in skeletal muscle (Hada et al., 2007). It is well known that exercise reduces the risk of T2D and CVD (Helmrich et al., 1991). Physical exercise makes a substantial improvement in lipid metabolism and insulin resistance in individuals with existing metabolic syndrome (Byberg et al., 2001). Recently Numao et al. demonstrated the effect of 60 minutes of

moderate intensity exercise on HMWA in young healthy males with high fitness level (Numao et al., 2008).

Accordingly; the aims of this study were to investigate the effects of a short bout of exercise on:

1- the plasma concentrations of HMWA and total adiponectin

2- the plasma concentrations of IL-6 and TNF-α.

3- the plasma concentrations of insulin and glucose.

These experiments were done in a group of normal healthy young women.

5.2 Materials and Methods

Volunteers were screened and enrolled for this study as described in chapter 2 section 2.2, following the inclusion and exclusion criteria in chapter 2 section 2.3.

5.2.1 Subjects

The College of Medical, Veterinary and Life Sciences Ethics Committee for Non-Clinical Research Projects, University of Glasgow approved this study. The information sheet about the study was given Appendix 2.

The volunteers gave written informed consent for taking part. Sixteen young healthy female volunteers were recruited for this study via posters assigned around the University campus. Thirteen subjects finished the whole experiment. The other three stopped for different reasons. One did not meet the inclusion criteria because she was significantly overweight. One stopped after the initial anthropometry and fitness tests, without giving any reason. One said she had a family problem.

The thirteen sedentary females were aged 20 to 30 years old. All of them were students at University of Glasgow, mostly European in origin. None of

these volunteers had participated in the earlier experiments. They confirmed that they did not do any vigorous exercise. Table 5-1 shows the general characteristics of volunteers. The volunteers were all in the follicular phase of their menstrual cycle.

	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m^2)	Fat (%)	$\dot{V}O_{2\,max}$ (ml/kg/min)
1	21	55.3	174.0	18.3	21.6	34.0
2	24	56.7	168.0	20.1	27.0	35.0
3	24	65.3	173.0	21.8	25.7	42.0
4	25	56.7	172.0	19.2	25.6	35.0
5	22	77.8	177.5	24.7	37.1	32.0
6	28	59.2	161.0	22.8	35.9	45.0
7	25	61.9	162.0	23.6	31.5	42.0
8	25	56.0	161.0	21.6	30.0	41.0
9	21	71.8	172.2	24.2	32.6	40.0
10	23	61.9	164.4	22.9	29.4	42.0
11	22	68.5	167.0	24.6	31.9	36.0
12	26	66.4	171.0	22.7	27.6	47.0
13	30	47.5	159.0	18.8	19.0	32.0
Mean ± SD	24.3 ± 2.7	61.9 ± 8.0	167.9 ± 5.9	21.9 ± 2.2	28.8 ± 5.2	38.7 ± 5.0

Table 5-1

Characteristics of the volunteers.

5.2.2 Design of the experiment

The general design was similar to that followed in chapter 3. Healthy female volunteers attended the lab on two days, rest on one and exercise on the other. The sequence was randomly allocated and the days were separated by at least two weeks.

Each day the volunteers arrived to the lab at 8 am having fasted overnight. A cannula was inserted in an antecubital vein to allow blood collection. A series of three 8 ml of blood samples were collected at intervals of one hour. The cannula was flushed with 8 ml non-heparinised saline solution after each collection to prevent clotting obstructing the cannula. Details of the technique are given in chapter 2 section 2.7.

On one day the volunteers exercised by walking on a treadmill at a preset speed. This ensured they exercised at 50% of their $\dot{V}O_2 max.$ That speed was determined at least one week before the actual experiment. Blood samples were collected directly before the exercise, at the end of the walk and one hour later. A fourth blood sample was taken two days later. The time line is shown in figure 5-1

On the other day the volunteers rested in the investigation room, seated on a chair or sleeping on a bed for the same duration. Four blood samples were collected at the same intervals.

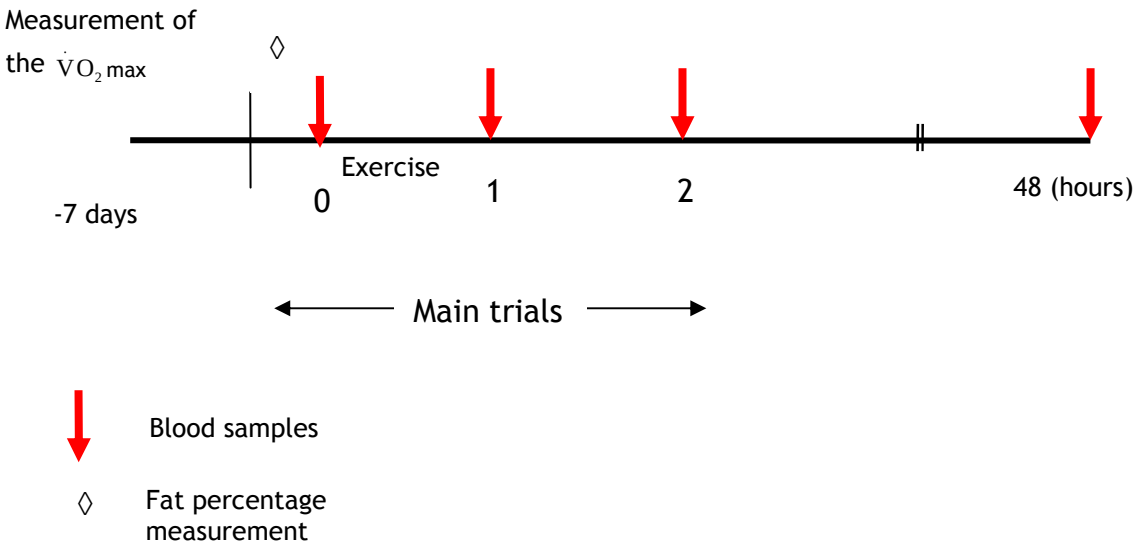


Figure 5-1

This diagram shows the experimental protocol. Maximum oxygen consumption was estimated for the all subjects at least one week before the main trials. Body fat percentage was estimated on the first day of the experiment. Blood sample collected at 0, 1, 2 hours. On exercise day, volunteers exercised for one hour at 50% of their $\dot{V}O_2 max$ after the 0 blood sample was collected. Volunteers came back 48 hour after the rest or exercise and one blood sample was collected.

5.2.3 Blood sample collection

The blood samples were collected using cannulae in EDTA tubes for subsequent measurement of adiponectin, HMW adiponectin, insulin, IL-6 and TNF-α. Additional samples for glucose measurement were collected in tubes with Fluoride Oxalate as the anti coagulant. All samples were centrifuged within 30 minutes of collection. Those to be used for measurements of HMWA, IL-6 and TNF-α were spun at 1000 g for 15 minutes at 4^{o}C (Sorvall, legend RT plus centrifuge, Germany). Those for measurements of the adiponectin, insulin and glucose were spun at 1400 g for 10 minutes (Universal 320R centrifuge, Germany). Plasma samples were transferred into two Eppendorf tubes using 1ml plastic Pasteur pipettes (Wilford Industrial Estate, Nottingham).

Plasma samples were stored at −80^{o}C for maximum 1 month. An exception to this was the storage of glucose samples which had to be kept frozen for about six months because of technical problem.

5.2.4 Statistical analysis

All data were analysed using statistical software (Minitab 16). Descriptive statistics were calculated for all parameters. Data were presented as mean ± SD. A normality test was applied to all parameters using the Ryan Joiner normality test. Paired t-tests were applied to compare the first samples on rest and exercise day. Two way repeated measures ANOVA (trial x time) were used to compare changes over time and across the two trials for normally distributed data. Non parametric Kruskal-Wallis test was applied for not normally distributed data.

5.3 Results

5.3.1 Adiponectin concentrations in plasma

The concentrations of total adiponectin and the HMW adiponectin were measured. The values for the first samples on the two days are plotted

below in figure 5-2. The values lie in the same range as in previous published works (Arita *et al.*, 1999; Hada *et al.*, 2007). The mean total adiponectin concentration was 10.2 ± 2.5 µg/ml and the mean HMW adiponectin concentration was 4.1 ± 1.7 µg/ml (Mean ± SD).

A paired t-test for normally distributed data showed no significant difference between the mean concentrations in the first samples on the two days (*P* value >0.05). The mean values for total adiponectin were 10.1 ± 2.4 µg/ml and 10.4 ± 2.7 µg/ml. The mean values for the HMW adiponectin concentrations were 4.2 ± 1.8 µg/ml and 3.9 ± 1.7 µg/ml for rest and exercise day respectively. These data are illustrated in Figure 5-2 A & B.

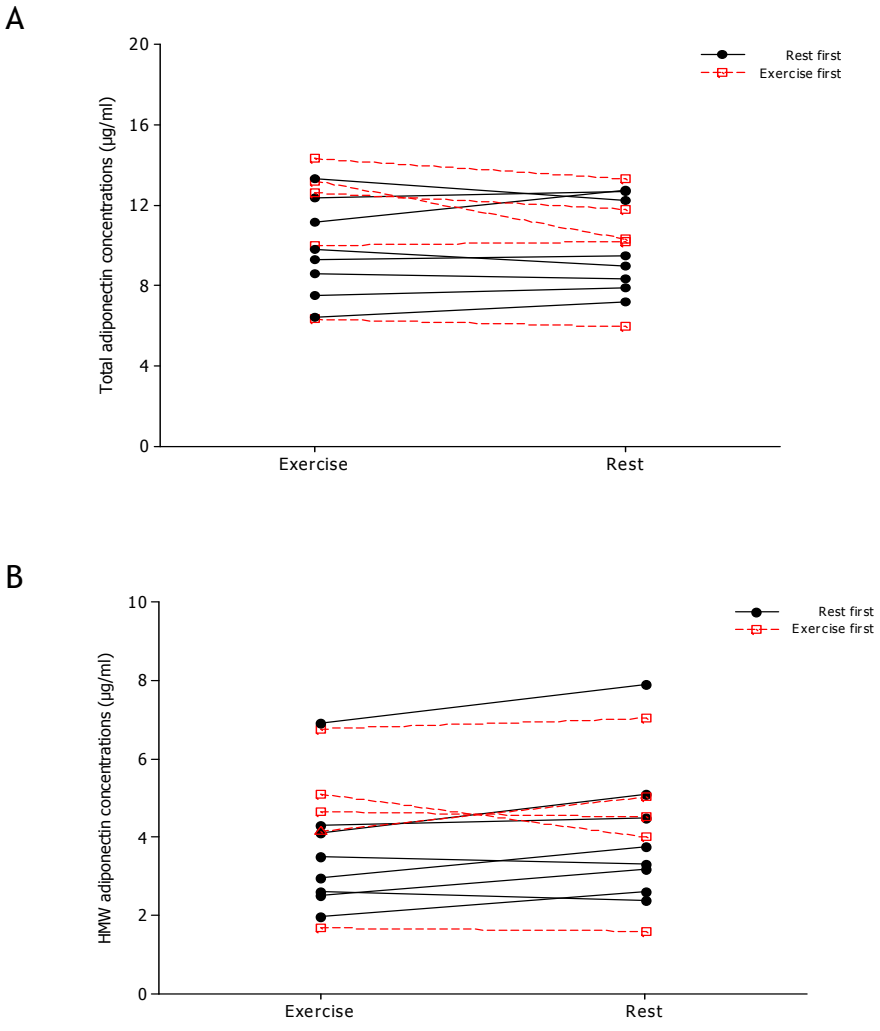


Figure 5-2

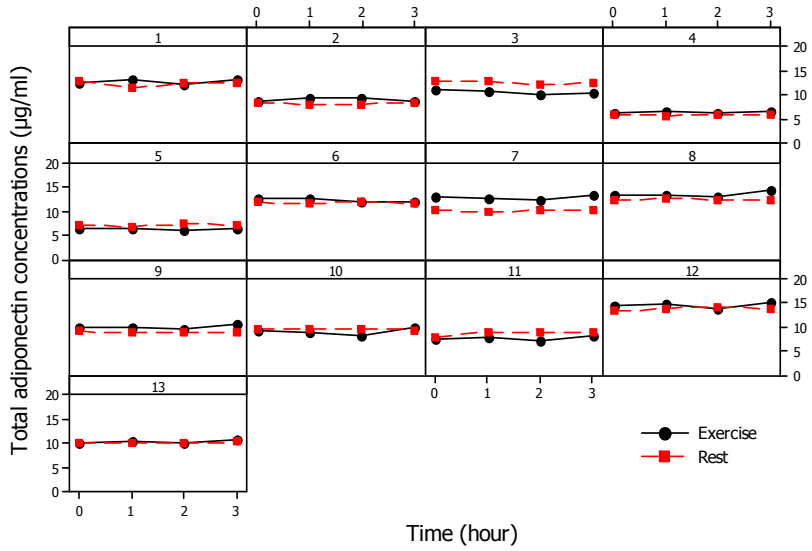
The interaction plots show total adiponectin concentrations (A) and HMW adiponectin concentrations (B) in the first samples for all volunteers on their rest and exercise days. ● The first day was resting day. □ The first day was exercise day

The plasma samples for each volunteer on both days were analysed at the same time. The interaction plots below illustrate the concentrations of total adiponectin figure 5-3 A, and HMW adiponectin concentrations figure 5-3 B. The overall impression is that neither concentration changes over the four samples.

Four series of samples had HMWA concentrations which were clearly higher than the others, figure 5-3 B. Those samples came from two volunteers on their rest and exercise days. They also had the highest total adiponectin concentrations. Similarly, the volunteers with the lowest total adiponectin concentrations also had the lowest HMWA concentrations.

The plots in figure 5-3 suggest that the data distribution might not be normal. This was tested using Ryan Joiner tests; figure 5-4 A and B show the results. The total adiponectin concentrations were normally distributed p values = 0.086 and the HMW adiponectin concentrations were not normally distributed *p* values <0.010. It is strange that no samples had concentrations between 5 and 6.5 µg/ml. This will have a big effect on the normality of these data. There is no obvious explanation for this. It is possibly just a chance observation that no samples had concentrations in that range.

A



B

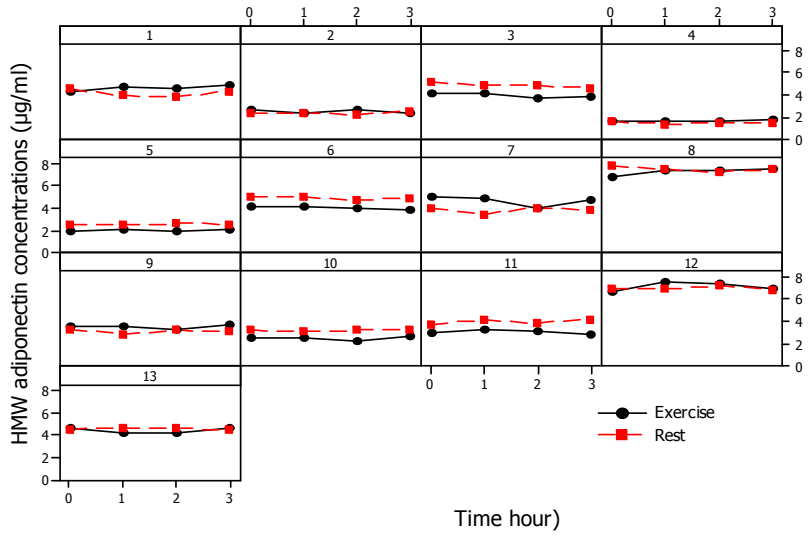
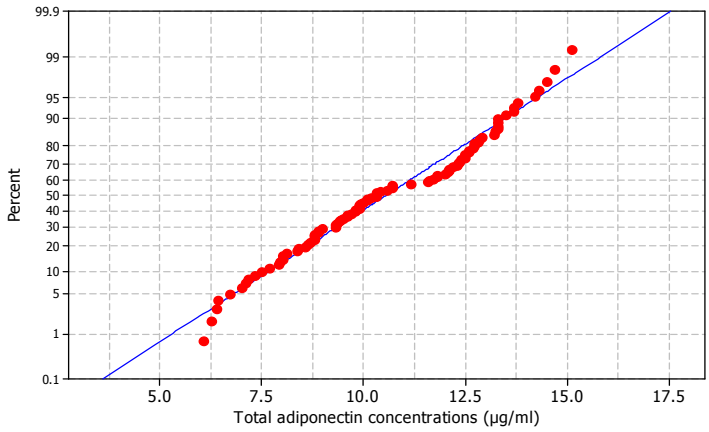


Figure 5-3

Panel A shows the total adiponectin concentrations for all volunteers at all sample time on both days. Panel B shows the HMWA concentrations in same blood samples. The values are stable over time.

A



B

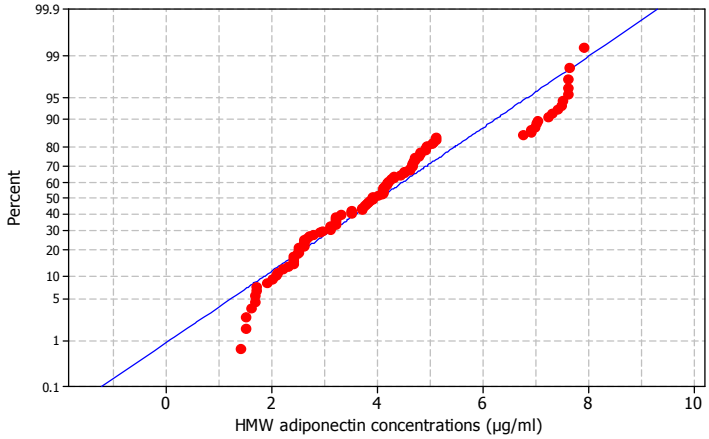
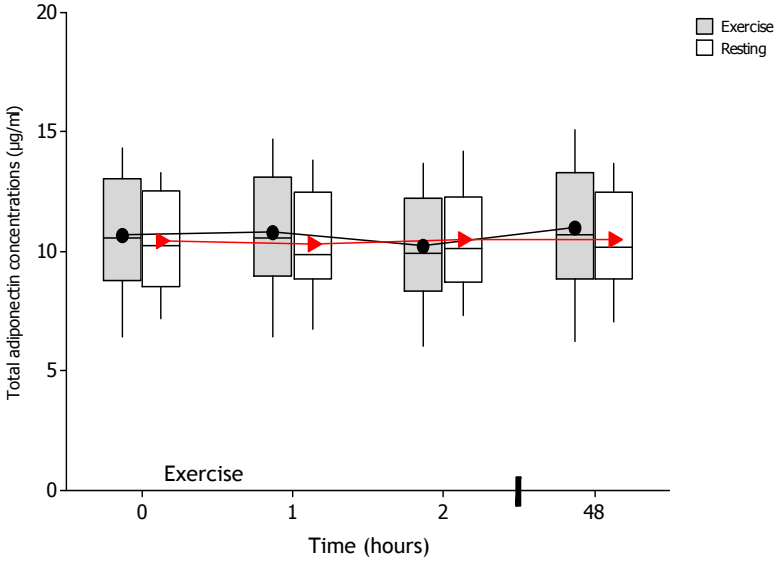


Figure 5-4 Normality test for total adiponectin concentrations (A), HMW adiponectin concentrations (B).

A two way ANOVA with repeated measures showed no time x trial interaction for total adiponectin concentrations figure 5-5 A (*p* value = 0.9). Nonparametric Kruskal Wallis test was applied for HMW adiponectin concentrations. No significant change was found between trails (*p* value = 0.5) figure 5-5 B. There was no change in total adiponectin concentrations and HMW adiponectin concentrations in plasma immediately, one, two, and 48 hours after rest and exercise. The statistical analysis confirms the early visual impression.

The percentage of the HMW adiponectin found in this study was 38.5% of the total adiponectin concentrations. There was no effect of the exercise on the ratio of HMW to the total adiponectin concentration.

A



B

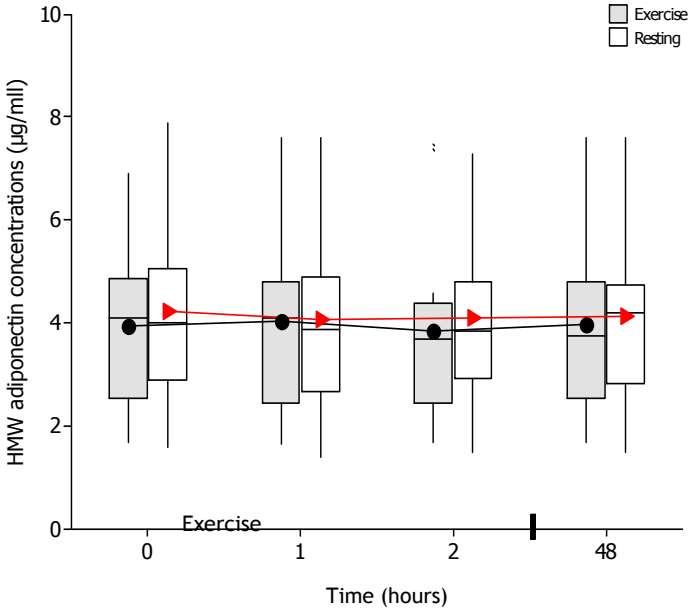


Figure 5-5

Box plot for plasma total (A) and HMW (B) adiponectin concentrations at rest and exercise. It is clear from the graphs that there is no effect of the exercise on both of the parameters *p* values > 0.05.

5.3.2 Plasma IL-6 concentrations

The samples from the thirteen volunteers was also analysed for IL-6 using the methods described in section 2-8-4. Two volunteers showed very high concentrations: one on their rest day and other on their exercise day. These values show as the outliers in the box plots of the data in figure 5-6A. Both

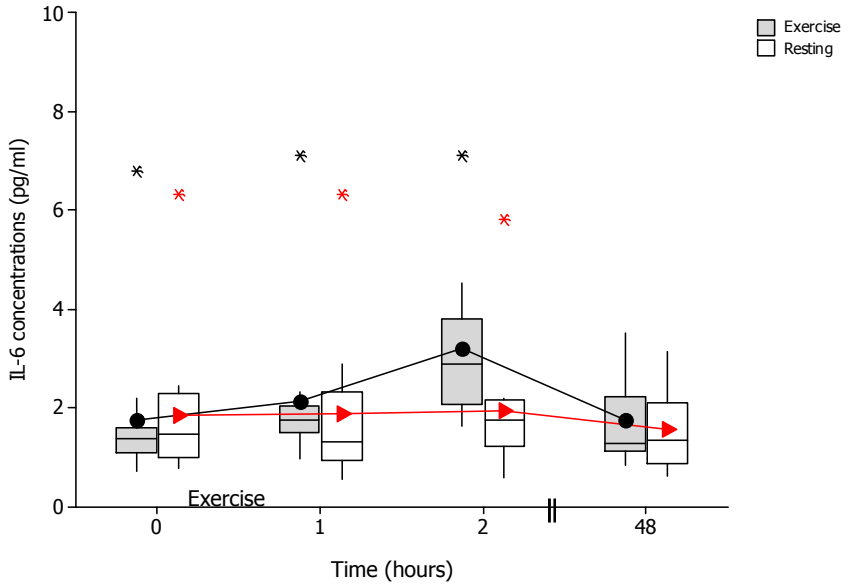
volunteers were unwell on these two days and reported that they had a cold. Consequently, those samples were omitted and were not included in the statistical analysis. Data for eleven volunteers were analysed.

The mean IL-6 concentrations in the first samples were 1.4 ± 0.6 pg/ml on the rest day and 1.3 ± 0.4 pg/ml on the exercise day. A paired t-test showed no significant difference between the mean concentrations first samples on both days (P value = 0.6). The data are plotted in Figure 5-7.

The scatter plot in figure 5-8 shows the IL-6 concentrations on rest and exercise days and 48 hours later. Almost all samples showed higher IL-6 concentrations one hour after the exercise.

The normality test was applied for the IL-6 data. Data were not normally distributed. Nonparametric test was applied, Kruskal Wallis test showed a significant change between trial for plasma IL-6 (*p* value = 0.036). This is clearly illustrated in the box plots in Figure 5-9.

A



B

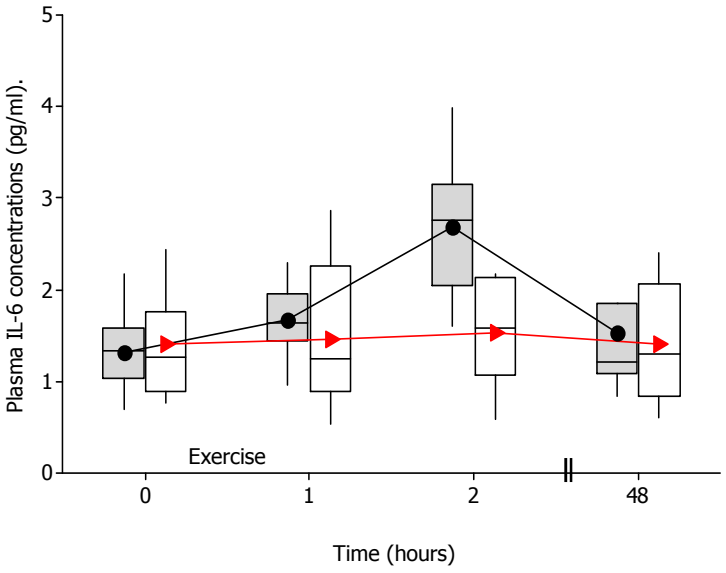


Figure 5-6

The figures show box plots to show the means of the raw data medians, quartile ranges and ranges of the IL-6 concentrations in plasma samples. The upper panel shows all data including the two volunteers with high samples. The starred values clearly lie outside the expected ranges. The lower panel shows the data re-plotted after removal of the six high samples.

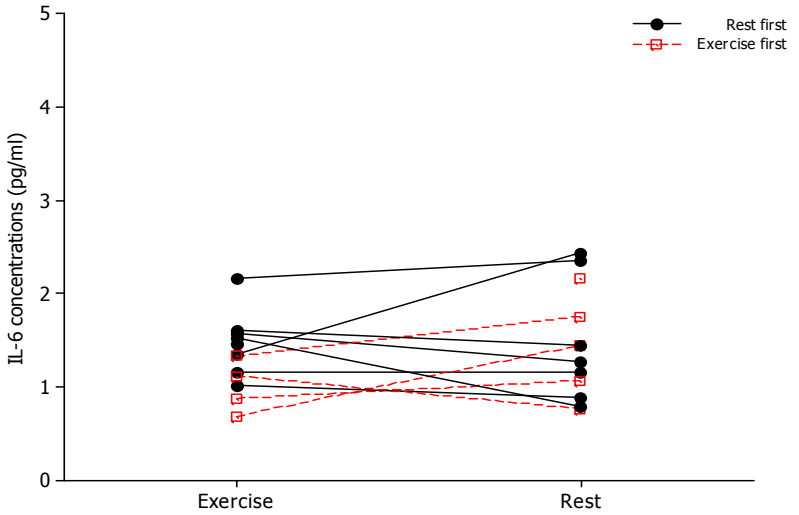


Figure 5-7

Interaction plot shows the concentration of IL-6 of the first samples on rest and exercise days. Most samples were stable. Two samples were higher on rest day than on exercise day. One sample was lower on rest than the exercise day. ● Volunteers were rested on first day. □ Volunteers were exercised on first day

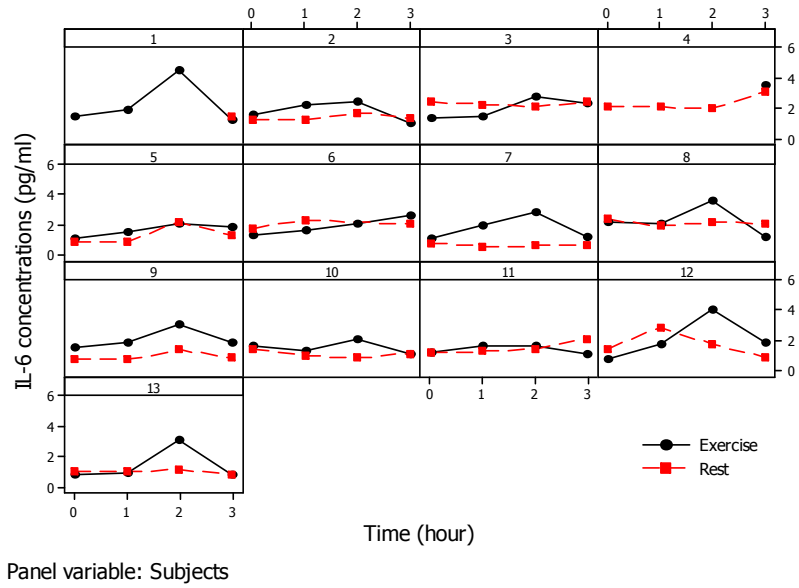


Figure 5-8

Scatter plot shows the IL-6 concentrations on rest and exercise days. Almost all samples show increased IL-6 concentrations one hour after the exercise. Two volunteers (1 & 4) have data for one day, as the missing data were plotted as outlier in figure 5-6

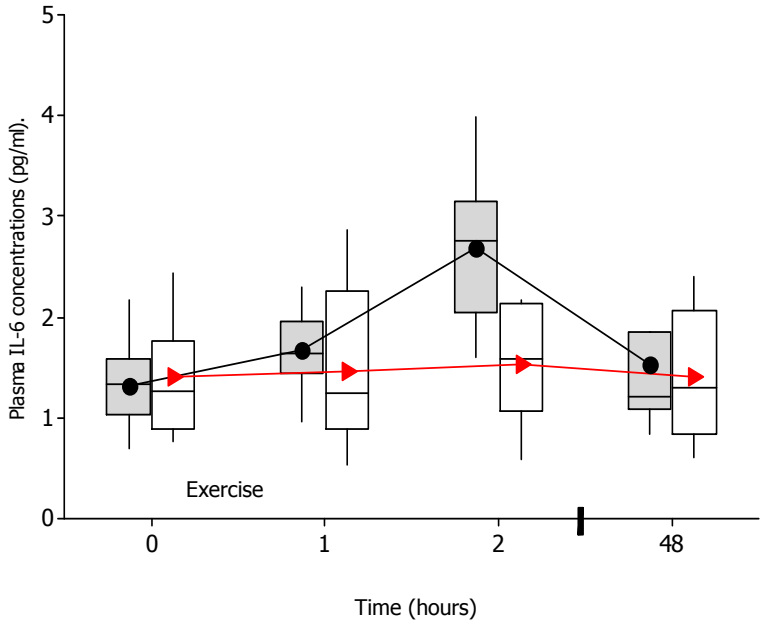


Figure 5-9

Figure shows the IL-6 concentrations on rest and exercise day. There is a significant increase of IL-6 concentrations one hour after the exercise (*P* value = 0.036).

5.3.3 Plasma TNF-α concentrations

The plasma samples were also analysed for TNF-α using the methods describe in chapter 2 section 2-8-4. The concentrations of the first samples on two days are plotted in figure 5-10. The mean values were 2.6 ± 1.5 pg/ml on rest days and 2.1 ± 0.9 pg/ml on exercise days. The result of a paired t-test showed no significant difference *p* value = 0.113.

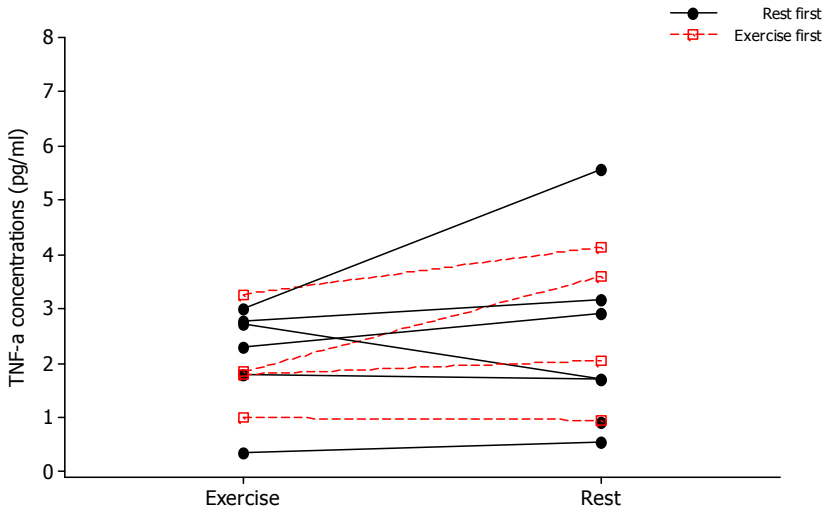


Figure 5-10

Interaction plot for the TNF–α concentrations show the first samples for the volunteers on rest and exercise days. Some samples showed no difference. Other samples showed lower values on exercise day. Only one sample showed higher value on exercise day. The data for ten volunteers, three volunteers had concentrations below the detection limit of the kit. ● Volunteers were rested on first day. □ Volunteers were exercised on first day

The scatter plot was plotted for the TNF-α for all the data. Big variations between the volunteers were illustrated. Some show no change, some show decreases in TNF-α concentration one hour after the exercise and the others show slight increases in TNF-α concentrations one hour after the exercise as illustrated in figure 5-11.

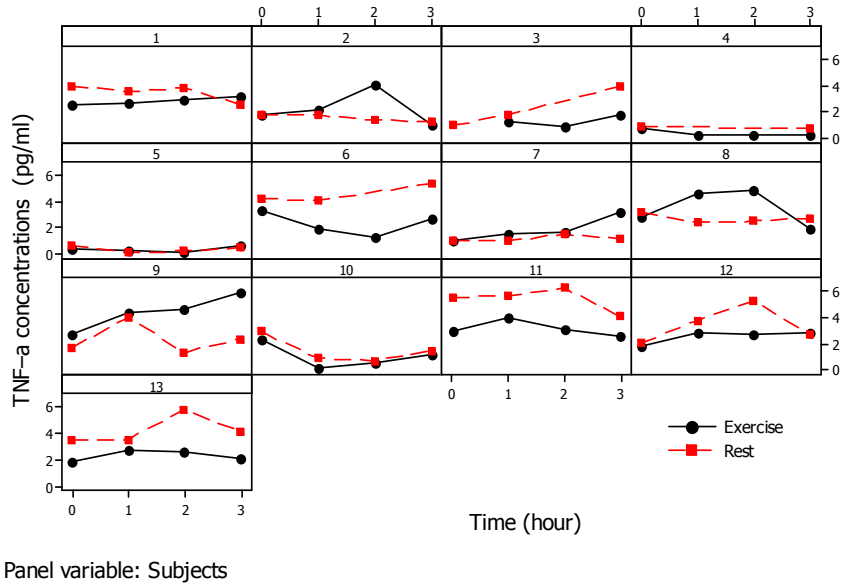


Figure 5-11 Scatter plot for TNF–α concentration on both days.

The normality test was applied for the TNF-α concentrations data. Data were not normally distributed (*p* value = 0.015). Nonparametric test was applied, Kruskal-Wallis test showed no significant change between trials (*p* value = 0.9) for TNF-α concentrations in plasma, Figure 5-12.

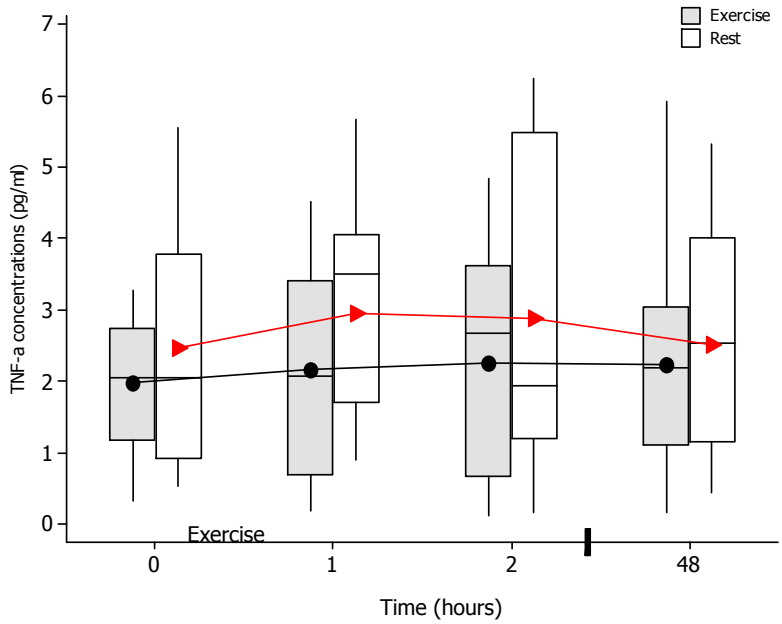


Figure 5-12

Box plot shows TNF-α concentration on rest and exercise days.

5.3.4 Plasma insulin concentrations

A normality test was applied to the insulin concentrations. It confirmed that the data were normally distributed.

The mean insulin concentrations for the first samples on rest and exercise day were 6.3 ± 2.3 mU/l. A paired t-test showed no significant difference between the first samples on rest and exercise day. The mean values were 5.8 ± 2.6 mU/l on rest and 6.7 ± 2.2 mU/l on exercise day (*p* value = 0.12). These values are plotted in figure 5-13.

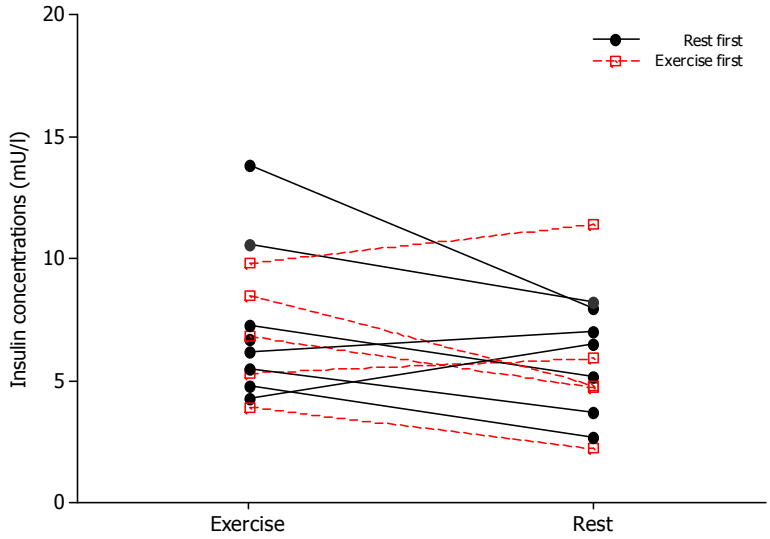


Figure 5-13

Interaction plot for the insulin concentrations in plasma shows the first samples for all volunteers on rest and exercise days. No significant difference between the first samples on rest and exercise day.

The scatter plot for insulin concentrations in plasma at all time points were plotted; this is shown at figure 5-14. Most samples showed sharp decrease one hour after the exercise.

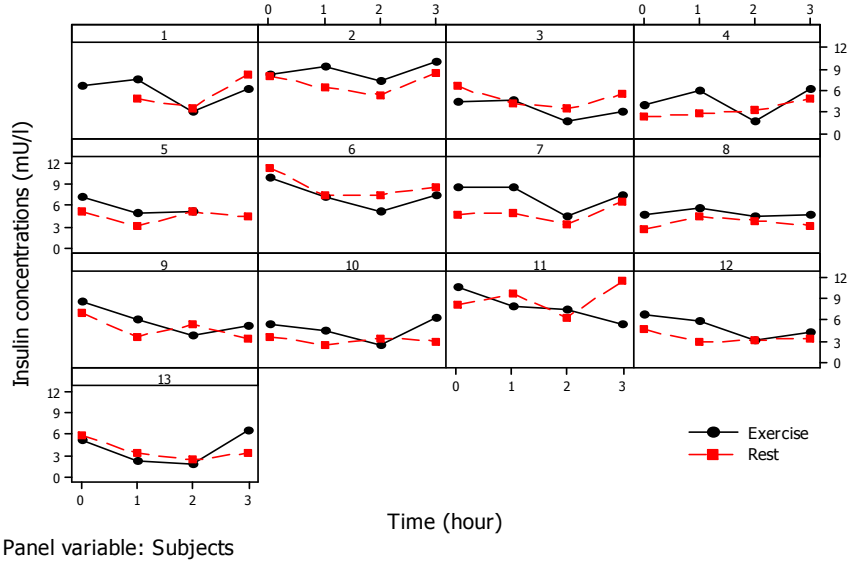
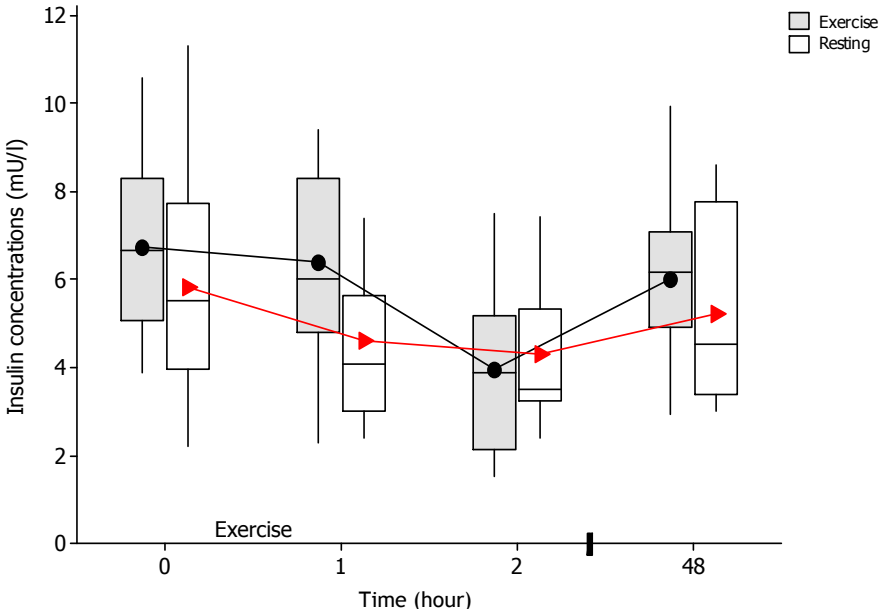


Figure 5-14

The scatter plot for the insulin concentrations at all time points. Almost all samples show decrease one hour after the exercise (time 2)

Two way ANOVA with repeated measures for insulin concentrations in plasma showed no time x trial interactions (*p* value = 0.3). These data are plotted in figure 5-15 A. However; one way ANOVA showed a significant difference with time in plasma insulin concentrations on the exercise day. Post hoc analysis using the Tukey method showed a significant difference in insulin concentrations one hour after exercise (*p* value = 0.003). This is clear in figure 5-15 B.

A



B

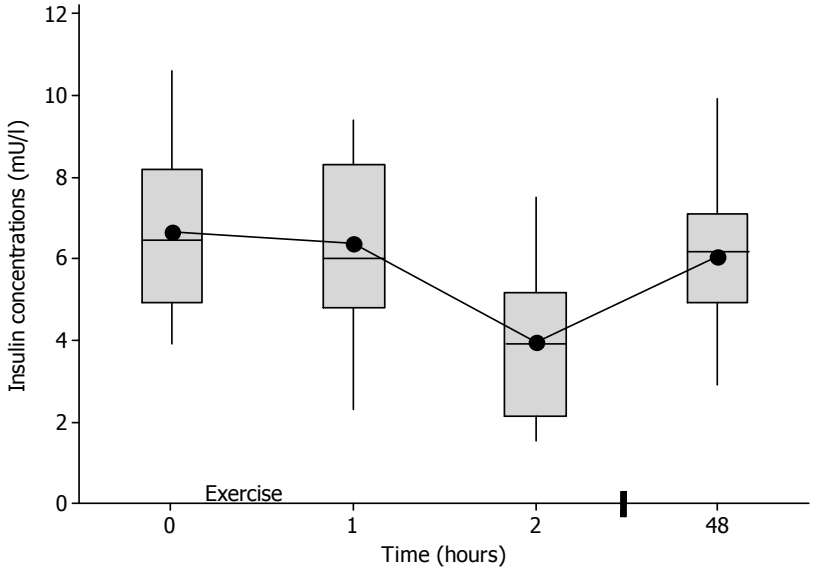


Figure 5-15

Box plot shows the insulin concentrations on rest and exercise days (A). It is clear from the graph that there is no significant difference in insulin concentration between rest and exercise days (*p* value = 0.3). The lower panel (B) showing the box plot for insulin concentrations on exercise day. A significant decrease of insulin concentrations occurs one hour after the exercise (*p* value = 0.003)

The lack of significant differences in the data plotted in 5-15A was surprising. Dr Tom Aitchison, formerly of the Statistic Department of Glasgow University was consulted. He suggested a further analysis to the differences in insulin concentrations between rest and exercise days (Exercise – rest). This method generates confidence intervals based on all differences (Newell *et al.*, 2010).

The differences in insulin concentrations between exercise and rest days at each time point for each person were calculated. The result for this analysis is plotted in figure 5-16. Figure 5-16B shows the difference of the time points after the exercise from the basal point or the time zero (before the exercise). The 98.3% CI of the differences were calculated and are plotted. The use of 98.3% CI is the result of a Bonferroni correction due to use of multiple comparisons

This analysis shows that there is a difference in mean insulin concentrations on two days. For example the mean concentration at start of sample collection is different on the two days by 1.4 mU/l, but the confidence interval of the difference includes zero (-0.9, 3.7) figure 5-16A. There is a drop in insulin concentrations at the 2 hour point. The insulin concentrations resting value are higher but the CI includes zero. The difference against resting time (time zero) is shown in figure 5-16B. The difference in insulin concentration is now clear, but it is CI still includes zero. The result suggests that a difference would have to be greater than 1.7 mU/l to be detected with this sample size.

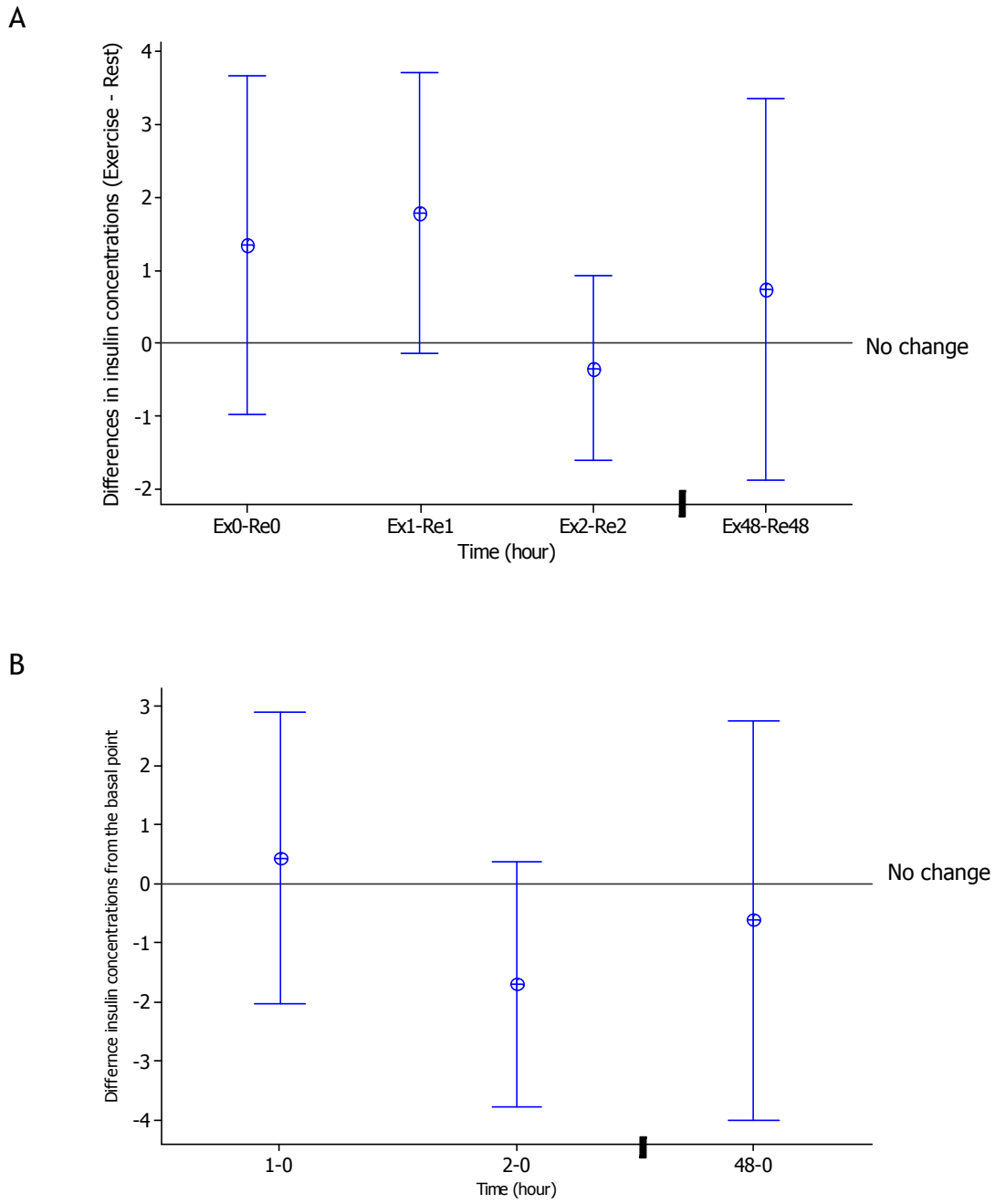


Figure 5-16

Interval plots show the difference in insulin concentration at different time points. A- Each point Shows the mean difference in insulin concentration (exercise – rest) at a series of time points: EX0-RE0 is the difference before exercise, EX1-RE1 is the difference one hour latter (immediately after the exercise), EX2-RE2 is the difference at 2 hours (one hour after the end of the exercise) and EX48-RE48 is the difference 48 hours latter. B- Shows the difference in insulin concentration at different time points after the exercise from the basal point (time before exercise): 1-0 is the difference at 1hour from the basal point, 2-0 is the difference at 2 hours from the basal point and 48-0 is the difference at 48 hours from the basal point. The mean difference is shown and the associated 98.3% CI. If the CI crosses the no effect line at zero, then there is no significant change.

5.3.5 Plasma Glucose concentrations

The mean glucose concentrations for the first samples on rest and exercise days were 4.3 ± 0.25 mmol/l. Paired t-tests showed no significant differences between the mean values on rest and exercise day. The mean values were 4.4 ± 0.2 mmol/l on rest and 4.3 ± 0.3 mmol/l on exercise day, *p* value = 0.4. These data are illustrated in figures 5-17 and 5-18.

The glucose concentrations in plasma were normally distributed. A two way ANOVA with repeated measures showed no significant time x trial interactions (*p* value = 0.6). The plasma glucose concentrations were similar on both days and did not change significantly with time. Box plots of these data are shown in figure 5-19

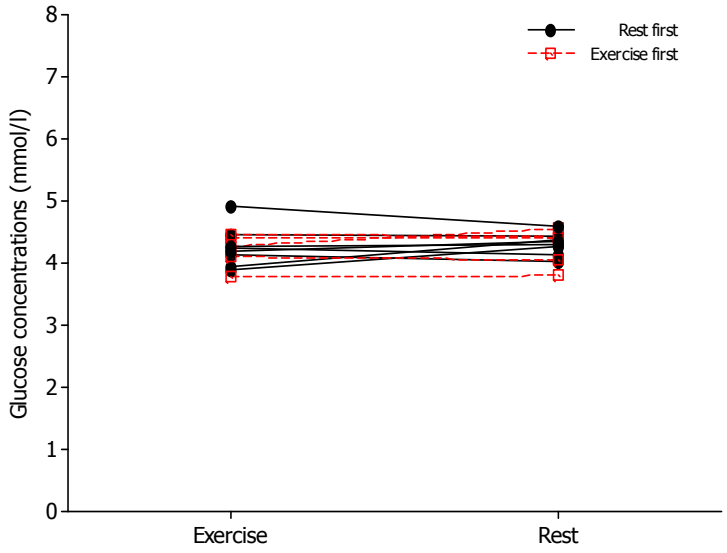


Figure 5-17

Interaction plot for the glucose concentrations in plasma samples shows the first samples for all volunteers on rest and exercise days.

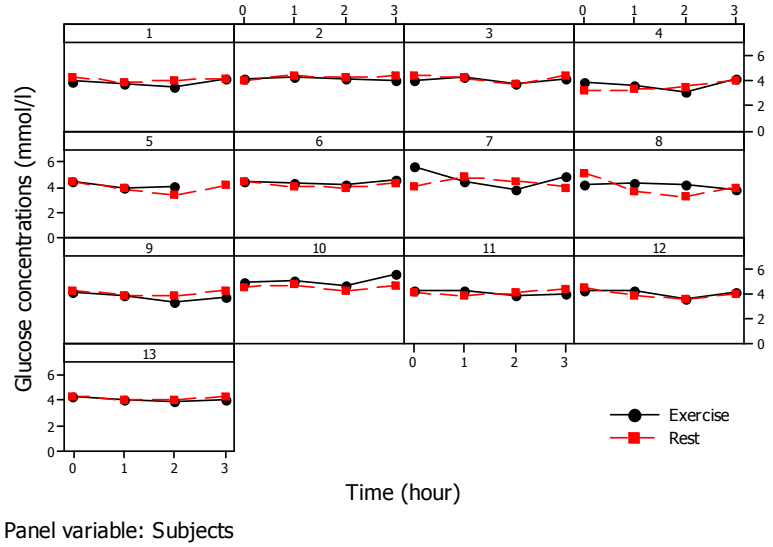


Figure 5-18

The graph shows the glucose concentrations for all volunteers at all time points. No differences in the concentrations were found.

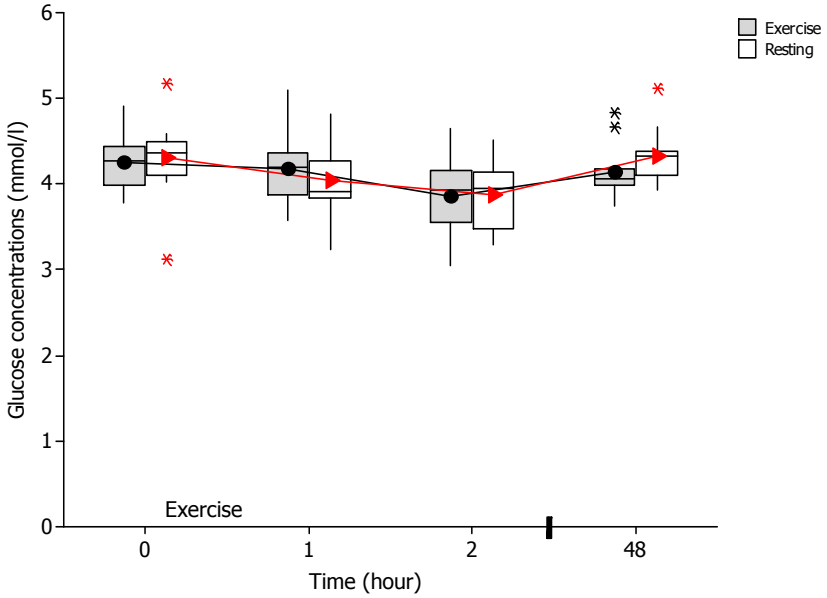


Figure 5-19

Box plot shows glucose concentrations on rest and exercise days**.** The (*) are outlier concentrations.

5.4 Discussion

This study was designed to investigate the effect of a short bout of exercise at 50% $\dot{V}O_{2\,max}$ on adipocytokines in healthy young females. The adipocytokines of particular interest were: adiponectin, both the total adiponectin and the HMW form, IL-6 and TNF α. In addition, the effects of exercise on insulin and glucose concentrations were investigated. These experiments follow on a similar approach described in chapter 3 where adiponectin was partially recovered by tissue microdialysis. The hope was that by moving to larger volume plasma samples it would be possible to investigate the full range of adiponectin and IL-6 and TNF-α which antagonise the actions of adiponectin.

This experiment gave more conclusive results than the first experiment using CMA microdialysis catheters. The volume of plasma samples was much larger, about 4 ml rather than 30-45 microlitres of dialysate. This made the handling of samples and the simultaneous analysis of several adipocytokines possible. The adiponectin concentrations were easier to analyse because they were much higher. The range of concentrations was similar to those found by other investigators. (Bobbert *et al.*, 2007; Hoffstedt *et al.*, 2004; Hojbjerre *et al.*, 2007; Hulver *et al.*, 2002; Jurimae *et al.*, 2009; Magkos *et al.*, 2010; Numao *et al.*, 2008). Also the concentrations for the other cytokines measured (IL-6 and TNF-α) were in the normal range and similar to others (Hojbjerre *et al.*, 2007; Kaminäski *et al.*, 2009; Lyngs *et al.*, 2002; Mohamed-Ali *et al.*, 1997; Nybo *et al.*, 2002).

It was easier to recruit new volunteers. The new group of volunteers were recruited in a much shorter period than that for the first experiment. It is possible that volunteers were more familiar with the idea of blood sampling than they were with the newer microdialysis technique. However, the duration of this experiment was longer and this did not seem to cause problems. The volunteers were invited to come during their follicular phase and that could be one to two weeks after the first visit.

The results in this chapter showed no significant effect of one hour of exercise on the total and HMW adiponectin concentrations in plasma, either directly after exercise or 48 hours later figure 5-5 A & B. These findings were in agreement with several previous studies on healthy young men, where moderate intensity exercise lasting 60-90 minutes, had no effect on the concentrations of HMWA or total adiponectin concentration (Numao *et al.*, 2008; Bobbert *et al.*, 2007; Hojbjerre *et al.*, 2007; Magkos *et al.*, 2010).

Other studies in young healthy men found no effect of higher intensity exercise (Kraemer *et al.*, 2003) or longer duration moderate intensity exercise (Punyadeera *et al.*, 2005). Furthermore, several studies concluded that adiponectin concentrations were not changed under the effect of chronic exercise lasting several weeks (Boudou *et al.*, 2003; Hulver *et al.*, 2002; Yatagai *et al.*, 2003) However, Kriketos et al showed that adiponectin concentrations more than doubled after 10 weeks of training of overweight males volunteers (Kriketos *et al.*, 2004).

One previous study has directly compared the effect of cycling at 65% $\dot{V}O_{2\,max}$ on adiponectin concentrations in both sexes and they too found no difference in concentrations after exercise in men or women (Ferguson *et al.*, 2004).

In summary, one hour of walking at 50% $\dot{V}O_{2\,max}$ has no effect on total or HMW adiponectin concentrations in plasma. This was similar to the finding of several previous studies.

IL-6 is a cytokine synthesised in many tissues including skeletal muscle. Muscle secretes the majority of the Il-6 in plasma during exercise (Steensberg *et al.*, 2000). However; other tissues such as, ligament and tendon related connective tissue and endothelial tissue in the vasculature are important sources of IL-6 during exercise (Langberg *et al.*, 2002). This secretion depends mostly on the duration and intensity of the exercise and is less affected by the mode of exercise (Fischer, 2006). In addition, it has been suggested that the increase in IL-6 concentrations is related to sympatho-adrenal response (Rhind *et al.*, 2001).

This study shows a significant increase of IL-6 concentrations after one hour of exercise figure 5-9. The concentration increased to almost double that of the basal level (1.3 ± 0.4 pg/ml to 2.9 ± 0.8 pg/ml). This finding was similar to others on male volunteers (Brenner *et al.*, 1999; Hojbjerre *et al.*, 2007; Kaminäski *et al.*, 2009; Lundby & Steensberg, 2004).The effect of the exercise on IL-6 has recently been reviewed by many researchers (Febbraio & Pedersen, 2002; Gokbel *et al.*, 2012; Lyngs *et al.*, 2002; Nybo *et al.*, 2002). One consistent finding of all these studies was a marked increase in circulating IL-6 concentrations after exercise. The data reported here are in line with this pattern. Interestingly, these observations on plasma Il-6 concentration changes are similar to those in microdialysis samples from subcutaneous abdominal adipose tissue (SCAAT) (Hojbjerre *et al.*, 2007). This suggests that Il-6, with a MW of 21 - 30 KD can be recovered successfully using their specialised microdialysis techniques with the large pore diameter membranes.

The data shown in figure 5-12 shows no change in the concentration of TNF-α after moderate exercise. The published literature suggests little effect of moderate exercise on TNF-α concentrations in plasma (Hojbjerre *et al.*, 2007; Numao *et al.*, 2011) in young men. The data in this thesis suggest that a similar lack of effect also exists in young women.

The effect of exercise on TNF-α concentration is relatively small in comparison with that on IL-6. These two cytokines are known as an inhibitors of the actions adiponectin (Fasshauer *et al.*, 2003). However the increased level of IL-6 concentrations in this study had no effect either on the total or HMW adiponectin. Thus, even if the concentration of adiponectin is stable after exercise, as shown in figure 5-5 A & B, its action may decrease as a result of raised IL-6 concentrations.

It is possible that the intensity or duration of exercise was too mild to provoke a response in adiponectin or TNF-α concentration. Helge et al. found an increase in IL-6 that was directly related to the intensity of the exercise (Helge *et al.*, 2003). The kinetics of hormones and metabolites, especially catecholamine, are increased by the intensity of the exercise and

that may have an inhibitory effect on adiponectin secretion in adipose tissue or even the gene expression of adiponectin (Cong *et al.*, 2007).

In short, in this study one hour of exercise increased the concentration of IL-6 significantly, but has no effect on the concentration of TNF-α. The role of IL-6 in insulin resistance is highly controversial (Petersen & Pedersen, 2005). In males, circulating IL-6 concentrations may or may not be associated with insulin resistance (Bastard *et al.*, 2002; Carey *et al.*, 2004). The concentration of insulin decreased in diabetic patients when they were given recombinant human IL-6. This indicated that the IL-6 improved insulin sensitivity (Petersen *et al.*, 2005).

The last part of this experiment examined the effect of exercise on insulin and glucose concentrations. However, the statistical analysis showed no difference between rest and exercise days on the insulin concentrations. The effect of the exercise on the insulin was very clear, as illustrated in figure 5-15. The initial ANOVA test showed no significant difference in insulin concentrations between rest and exercise day. This was unexpected, many papers have shown a clear effect of exercise on the insulin concentrations. This prompted the further analysis of the differences shown in figure 5-16. The most likely explanation is that a fall in mean insulin of less than 1.7 mU/l did not show up as a significant, because the 98.3% CI included zero. The interval was raised from 95% to 98.3% to compensate for repeated tests. This is similar to Bonferroni correction. The author suspects that a larger sample size would have given a more robust estimate of the CI, and thus might allow the change to be identical as statistically significant. Thus the study was probably under powered. The number of volunteers could not be increased because of the difficulty in the recruitment of volunteers and the time and financial limits of the study. Interestingly Hojbjerre et al. also found no effect of the exercise on insulin concentration (Hojbjerre *et al.*, 2007). These experiments used similar number of volunteers and may have encountered the same statistical problem. At the same time there was no effect of one hour of the exercise on the glucose concentrations.

During moderate duration of exercise, plasma glucose concentrations remain quite stable due to the increase in hepatic glucose output (Romijn *et al.*, 1993). Kjaer states that 'hepatic glucose output is stimulated by afferent neural feedback from contracting muscle and by unidentified substances released from the muscle into the blood'. Consequently, a decline in insulin concentrations in plasma is the most important factor stimulating the production of glucose from the liver (Kjaer, 1998).

The data in this chapter are in agreement with several studies of the effects of acute exercise on plasma insulin and glucose concentrations where the general effect is an increase in insulin sensitivity after exercise. (Bobbert *et al.*, 2007; Ferguson *et al.*, 2004; Hulver *et al.*, 2002; Magkos *et al.*, 2010; Numao *et al.*, 2008; Yatagai *et al.*, 2003).

Most of the studies on the effect of acute exercise on total and HMW adiponectin concentrations in plasma were on young healthy males (Bobbert *et al.*, 2007; Hojbjerre *et al.*, 2007; Kriketos *et al.*, 2004; Numao *et al.*, 2008; Punyadeera *et al.*, 2005; Yatagai *et al.*, 2003). This experiment was on young healthy females. However; the finding was similar to that of the previous studies. The exercise has no effect on total and HMW adiponectin concentrations. IL-6 concentrations increased to double concentration of the basal level and insulin sensitivity was slightly improved.

Chapter 6

General discussion

6.1 Summary

The prevalence of obesity is a rapidly increasing problem all over the world. It is a matter of concern due to the associated serious risks to health. It is a natural consequence of over nutrition and sedentary lifestyle. However, the main causes are not well understood. Obesity is commonly related to insulin resistance, abnormalities in the metabolism of glucose and T2D (Iacobellis & Leonetti, 2005). Adipocytokines are thought to play a vital role in the link between obesity and pathophysiology of diabetes (Snijder *et al.*, 2006). These adipocytokines include leptin, TNF-α, IL-6, adipsin, resistin, visfatin and adiponectin (Ronti *et al.*, 2006).

There is a significant difference in the occurrence of obesity between sexes all over the world (Chalfant *et al.*, 2012). It is more common among women. Further , it is recognised that " the prevalence of overweight and obesity was higher in economically developed countries compared with economically developing countries in 2005" (Kelly *et al.*, 2008). Adiponectin is an adipokine secreted mainly from adipose tissue. It has inverse relation with obesity and insulin resistance, as the obese people tend to have lower adiponectin concentrations than lean.

It is well known that exercise increases insulin sensitivity and insulin is one of regulators of adiponectin concentration. This thesis aimed to investigate the effect of acute exercise on the adiponectin concentration in young healthy lean and overweight females.

The samples used in the experiments in this thesis were obtained in two main ways. Dialysate samples (chapter 3 &4), and plasma samples (chapter 4 & 5). Dialysate samples were collected from abdominal subcutaneous tissue using CMA 66 microdialysis catheters. There is a long history of analysis of plasma

samples. In contrast, microdialysis is a relatively new technique first used in 1985 (Ungerstedt, 1991). It is a validated sampling technique that allows direct measurements of exogenous and endogenous substances in the interstitial fluid in vivo (Clough, 2005). One of the advantages of this technique is the possibility of follow the regional variations of the molecules of the interest at the site of attention. Recently, it is possible to recover the large molecular weight molecules using microdialysis catheters with high cut off membranes (Clough, 2005).

The advantages of the dialysate samples over the plasma samples were the clean samples. Dialysate samples need no preparations, it is ready to store or analyse without any extra work. In contrast, the plasma samples may include proteins and enzymes. One possibility is that enzymes may affect the concentrations of the molecules being studied. Some authors have added protease inhibitors such as Trasylol to prevent this (Hojbjerre *et al.*, 2007; Nielsen *et al.*, 2009). In addition, blood samples require processing such as centrifugation to separate the plasma from the cells. Despite this, there were advantages in using plasma samples rather than dialysate. Firstly, the plasma contains all the forms of adiponectin. In addition it is easy to collect larger volumes and so several assays could be run from one sample.

The initial experiments reported in chapter 3 examined adiponectin concentrations in subcutaneous adipose tissue. It was suspected that this was the major site for adiponectin secretion (Scherer *et al.*, 1995). The experiments used the commercially available CMA 66 microdialysis catheters with cut off 100 KD. In general these catheters worked well and were well tolerated by the volunteers. However, two problems were experienced: the volumes of recovered fluid were very small and very low adiponectin concentrations were found. This made the interpretation of the results difficult. The analytical procedure was good. This is shown by the inter assay coefficients of variation. These were 1.7% and 1.8% respectively for low and high controls. While the intra assay coefficient of variation for low and high controls were 3.1% and 2.2% respectively. The sensitivity of the ELISA kits was high. In conclusion, after review of the balance of benefits and problems

associated with the microdialysis approach it was decided to move to recovering plasma samples.

The second study was aimed to compare the dialysate and plasma adiponectin concentrations in samples taken at the same time. The second study found substantial differences between the plasma and the interstitial adiponectin concentrations. The plasma concentrations were up to 1791 times greater (Table 4-2). Thus, a third series of experiments was designed with the aims of studying the effect of acute exercise on the concentrations of total and HMW adiponectin in plasma. In addition, the effect of exercise on other cytokines such as IL-6 and TNF-α and the effect on insulin and glucose concentrations were examined.

6.2 The main findings

Successful recovery of the fluid over two successive days. CMA microdialysis catheters were great technique to work with.

No difference in adiponectin concentration in dialysate samples between lean and overweight females.

The concentration of adiponectin was very low due to the polymerization of adiponectin to bigger forms that have molecular weights bigger than the cut off of the microdialysis membranes.

No clear effect of the exercise on the concentration of adiponectin in dialysate samples (chapter 3).

The second study showed a substantial difference between plasma and dialysate adiponectin concentrations (chapter 4). That confirms the low recovery of the 100 KD CMA microdialysis catheters.

A single bout of exercise at 50% $\dot{V}O_{2\,max}$ had no effect on total and HMW adiponectin, TNF-α and glucose concentrations in plasma in young healthy females (chapter 5).

IL-6 concentration increases to double of the basal values after one hour of the exercise at 50% $\dot{V}O_{2max}$ in young healthy females (chapter 5)

The effects of exercise on insulin concentrations were not clear; the insulin concentration showed a significant decrease one hour after the exercise when tested with one way ANOVA. The effect was not significant when tested with the two way ANOVA with repeated measures and differences. Subsequent analysis suggests that the change seen was at the limits of what might be detected with this sample size.

On the bases of the findings of the three studies in this thesis it was interesting to work with the microdialysis system, despite of some problems such as the small sample volumes. As mentioned before the volume of the samples collected was 30 – 45 microlitres. To overcome this problem more than one catheter could be used per volunteer, or the time of the collection of single sample could be increased to more than one hour. The previous studies used more than one catheter at the same time (Hojbjerre *et al.*, 2007; Nielsen *et al.*, 2009). The advantages of plasma samples overcome this problem in this thesis.

The concentrations of adiponectin in dialysate samples were very low (chapter 3 & 4) and very variable, Figure 3-5B. However, Hojbjerre et al. demonstrated that the concentrations of adiponectin in SCAAT of lean and overweight subjects, obtained using a microdialysis catheter with 950 KD cut-off, is about 20% of the plasma adiponectin concentrations (Hojbjerre *et al.*, 2007). “Adiponectin circulates in plasma as complexes of which some are larger than 400 KD” (Pajvani *et al.*, 2003b). Therefore, the adiponectin complexes could be too large to pass the microdialysis membrane, and the actual interstitial adiponectin concentration could be higher than that measured by the microdialysis probe.

One possible reason for the low concentrations of adiponectin found in SCAAT, even when the most permeable 950 KD membrane has been used, could be the formation of larger complexes of adiponectin in the interstitial

fluid. Secondly, adiponectin is secreted from other adipose tissue stores like visceral adipose tissue. Then, adiponectin is passed in vesicles into the interstitial space (Hojbjerre *et al.*, 2007). Next, adiponectin is strongly bound to transport proteins in plasma that lead to a low free plasma concentrations of adiponectin in balance with the interstitial adiponectin concentration. Another possible reason is that the transport of adiponectin from adipocyte to plasma is an active regulated transport process working against the concentration gradients. Or adiponectin is transported from the interstitial fluid by lymph (Hojbjerre *et al.*, 2007).

These findings led to the second study which confirmed that the problem was with the cut off of the CMA microdialysis catheters. Consequently it was decided in the last study to work on plasma samples (chapter 5). The first idea in this chapter was to investigate the effect of acute exercise on the high molecular weight adiponectin instead of the total adiponectin. Several studies had been done on the effect of the exercise on the total adiponectin concentration. However the results were controversial but the majority demonstrated no effect of the acute and chronic exercise on the total adiponectin concentrations. In addition very few studies were on the effect of the HMW adiponectin concentrations as the biologically active form of adiponectin (chapter 5).

There were no effects of acute exercise on total or HMW adiponectin concentrations in plasma, either directly or after the two days follow up. The exercise was moderate (50% $\dot{V}O_{2\,max}$) and for a short time (60 minutes) without any specific diet control. The high concentration of adiponectin in plasma (3 -30 µg/ml) has been mentioned in many studies. This concentration is relatively stable over the day (Gavrila *et al.*, 2003). This elevated adiponectin concentration and stability might need a big alteration in the rate of secretion (Hojbjerre *et al.*, 2007). Previous studies demonstrated that weight loss or reduction in body fat is necessary to increase adiponectin concentrations in plasma (Hotta *et al.*, 2000). That might be achieved by a longer exercise program with a controlled diet. Also there was no effect on the ratio of HMW to the total adiponectin concentration. The percentage of the HMW adiponectin found in this study was 38.5% of the total adiponectin

concentrations. This was similar to the previous study (Beltowski *et al.*, 2008).

In comparison between the adiponectin concentrations in plasma for males (chapter 4) and females (chapter 5), females have higher adiponectin concentration than males. However the two groups were not different in the concentration of adiponectin in dialysate samples. The higher plasma adiponectin levels in women were in agreement with many other studies (Arita *et al.*, 1999; Cnop *et al.*, 2003; Pajvani *et al.*, 2003a; Steffes *et al.*, 2004). This finding might be related to androgen levels since androgens can have an inhibitory effect on adiponectin secretion. Testosterone suppressed adiponectin secretion in mice and in cells in culture media (Nishizawa *et al.*, 2002). In contrast, the effect of oestrogens on adiponectin secretion is not fully understood (Sieminska *et al.*, 2005).

No correlations were found between total and HMW adiponectin concentrations in plasma and BMI or fat percentage that might be because the volunteers had similar BMI and fat percentage. A strong negative correlation was illustrated between the BMI and adiponectin concentrations by others (Arita *et al.*, 1999; Weyer *et al.*, 2001). Although this correlation was not found in Hojbjerre's study. Also the group were from same age group so no correlation between age and total or HMW adiponectin concentrations was seen. It might be the sample size is small to detect a significant correlation, although, the sample size is comparable to most published studies. Again despite the positive effect of the acute exercise on IL—6 (chapter 5), there was no detectable inhibitory effect of that increase on total or HMW adiponectin concentration.

Insulin concentrations showed a significant decrease using a one way ANOVA but no significance using a two way ANOVA with repeated measures and differences. This may indicate that the result is close to significance but the small sample size makes it difficult to be certain. Glucose concentrations show no change. This finding was similar to that which has been described by Hojbjerre et al (Hojbjerre *et al.*, 2007)

6.3 Conclusion

The results in this thesis found that the CMA 66 microdialysis catheters with a cut-off of 100 KD were not enough or appropriate to recover the total adiponectin or its active form. The positive effect of acute moderate exercise on IL-6 and insulin had no effect on total and HMW adiponectin concentrations in healthy young females. Whether this effect is applicable to overweight and obese volunteers is not clear.

6.4 Difficulties

It was difficult to recruit volunteers for these studies. That might be understandable especially for the application for the microdialysis catheters. A previous study on the dialysate adiponectin concentrations demonstrated increased interstitial adiponectin concentration despite no change in adiponectin concentrations in plasma for the same group of volunteers (Hojbjerre *et al.*, 2007). That was interesting but the limitation of the present study was that catheters with higher cut-offs could not be bought or manufactured locally.

6.5 Future studies

This study shows the effect of acute moderate intensity exercise on total adiponectin concentration, HMW adiponectin concentration, IL-6, TNF- α, insulin and glucose in young healthy females. However little is known about the effect of the same activity on these hormones on over weight and obese females. It would be good for the future studies to be on this group of volunteers. Further, some studies reported ethnicity difference in adiponectin concentration, and most of the published studies were on limited ethnic groups. So it would be interesting to investigate other ethnic groups.

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Appendix 1

Application Form for Ethical Approval

Ethics Committee for Non Clinical Research Involving Human Subjects, Material or Data

Application Form for Ethical Approval

NOTES:

A submission to this Committee does not automatically result in approval. Investigators must wait for written approval before commencing data collection. Disciplinary measures will be taken if work commences without ethical approval being in place. The matter will be referred to the Dean for appropriate action.

THIS APPLICATION FORM SHOULD BE TYPED, NOT HAND WRITTEN.

ALL QUESTIONS **MUST** BE ANSWERED. "NOT APPLICABLE" IS A SATISFACTORY ANSWER WHERE APPROPRIATE.

Project Title: An investigation of the effects of short bouts of exercise on adiponectin in young healthy females.

Is this project from a commercial source, or funded by a research grant of any kind?

No

If yes,

a) Has it been referred to Research & Enterprise?

Has it been allocated a project Number?

b) Give details and ensure that this is stated on the Informed Consent form.

Date of submission: 27 January 2010

Name of all person(s) submitting research proposal
Mrs. Mabroukah Alzwayi, Dr Ronald Baxendale

Position(s) held: PhD. Student (IBLS), Reader

Division: Integrative and system biology (ISB)

Address for correspondence relating to this submission
Mrs Mabroukah Alzwayi, room 244a, West Medical Building, Glasgow University

Name of Principal Researcher (if different from above e.g., Student's Supervisor):
Dr Ronald Baxendale

Position held: Reader_____

1. Describe the purposes of the research proposed.

Obesity is a major health problem in most countries. A great amount of advice on diet and physical activity is available, but the details of the effects of physical activity and the hormonal control of adipose tissue remains poorly understood. Adipose tissue is not only energy storage organ, but also it plays a major role in the secretion of many important metabolic hormones. These are known as adipokines (1). Some of these adipokines play an important role in metabolic syndromes such as type 2 diabetes and related cardiovascular complications. Central or visceral obesity (2) is particularly important.

The purpose of this project is to investigate the effect of acute exercise on adiponectin and resistin concentrations in both plasma and interstitial fluid in young healthy women. The effects of exercise on the concentration of adiponectin have been examined in recent studies in healthy young males, but conflicting results have been reported (Stenken *et al.*, 2010).

Some studies conclude that chronic exercise has no effect on plasma adiponectin concentrations (4, 7). However, others found that exercise increased the level of plasma adiponectin concentration substantially but only after two to three bouts of moderate intensity exercise. This last study was performed in overweight males (5).

Hojbjerre et al (8) used a microdialysis technique to compare interstitial fluid and plasma concentrations of adiponectin. They showed that adiponctin concentrations increased in interstitial fluid in adipose tissue after acute bout of exercise in both overweight and lean male subjects; on the other hand, there was no effect on the plasma adiponectin concentration. Perhaps the adipokine concentrations are diluted in the larger plasma volume.

This proposal will follow the protocol used by Hojbjerre et al. We plan to repeat their study in healthy young women.

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2. Please give a summary of the design and methodology of the project. Please also include in this section details of the proposed sample size, giving indications of the calculations used to determine the required sample size, including any assumptions you may have made. (If in doubt, please obtain statistical advice).

The design of the experiment follows the establish protocol published by Højbjerre et al (2007) except that healthy female volunteers will be used rather than the healthy males in their study.

We seek permission to invite up to 16 healthy adult females (8 lean and 8 overweight) in the age range 20-35 years. Volunteers will be invited to the lab three times.

First day, the visit will take about 1 hours. The experiment will be explained, questions will be answered and consent will be sought. They will know they are free to stop the experiment at any time. If they agree to participate, they will familiarize with the lab. Their age, weight, height, $VO_{2\ max}$, total body fat percentage and abdominal skin fold thickness will be measured.
Lastly, the volunteer will be randomly assigned to the following two days. These will be either exercise day followed by rest day or rest day followed by exercise day. For simplicity only the first option is described.

Second day: they will be invited for exercise day. They will visit the lab for 6 to 8 hours. They will arrive in the morning fasted. A microdialysis catheter will be inserted into their subcutaneous fat about 4 cm lateral to the umbilicus on the left side for interstitial fluid collection and another catheter will be inserted into the antecubital vein for blood collection. Standard aseptic precautions will be followed. The volunteer rests for 3 hours, exercises for 1 hour at about 50% of their maximal capacity by walking on a treadmill and then rests again for 2.5 hours.

Third day is the rest day. This day lasts for 6 hours. During this day the volunteer follows the same protocol as the exercise day except that exercise is omitted.

These blood and interstitial fluid samples will collected every 30 minutes and subsequently the adiponectin, interlukin-6, insulin and glucose will be measured. The concentrations before, during and after exercise will be compared using two way repeated measures ANOVA.

3. Please give a detailed account of the method of recruitment of volunteers.

In the first instance, will attempt to recruit women volunteers from students and staff of Glasgow University. They will be in the age range 20 – 35 years. Initial contact will be via posters, websites and personal contact. If this fails, the search will be extended to other universities in the west of Scotland.

We aim to include healthy young women who are non smokers. We aim to exclude any pregnant volunteers or any volunteers who likely to become pregnant. We also aim to exclude any woman who has a first degree relative with type 2 diabetes.

4. Describe the research procedures as they affect the research subject and any other parties involved.

The volunteers will be invited for three visits, the first one lasts up to 4 hours to familiarize volunteers with the experiment, to distribute information sheet, to give an oral explanation of the nature of the experiment and answer any questions. After that the volunteers will be asked to sign the consent form if they are agreeing to participate in the experiment. Volunteers will complete a PARQ to confirm they are in good health before testing begins.

Then base line measurements will be made. Age, weight and height will be measured. Abdominal skin fold thickness will be measured with callipers. Total body fat will be measured by bio impedance. This requires the attachment of adhesive pads to the hands and feet. The volunteer must lie flat for 15 minutes. Their $VO_{2\ max}$ will measured by a standard treadmill test during which their heart rate will be measured.

The maximum oxygen up take (VO_{2MAX}) is the utilization of oxygen at maximum rate is a commonly used measure of aerobic fitness. We propose to establish this by extrapolation from sub-maximal values taken during low and moderate intensity exercise.

The volunteer's heart rate will be recorded continuously using a Polar Heart Rate monitor. The heart rate will be recorded as a running average over 10 second periods. The volunteer will inspire room air via a Hans Rudolph valve. They will wear a nose clip, and will be allowed to rest for about five minutes to become familiar with the equipment. The volunteer's expired gas will be collected in a 150L Douglas Bag via the expired gas port of the Hans Rudolf valve via flexible tubing and a T piece valve. Gas will be collected for periods of 2 minutes. Their oxygen consumption in each 2 minute period will be calculated using standard formulae using the volume and composition of the expired gas sample. Appropriate corrections will be made for gas temperature and barometric pressure.

The oxygen consumption will be measured at rest, and a low intensity exercise with a mean heart rate of about 110 beats/minute, at a higher intensity with a mean heart rate of about 130 beats/ minute and finally at a mean heart rate of about 150 beats/minute. Thus, even at the highest intensity of exercise the heart rate will not exceed 80% of their predicted maximum. If the volunteer is unhappy with the exercise intensity, the test will be terminated. The exercise intensity will be controlled by adjusting the speed and gradient of the treadmill. The resting value will be taken with the volunteer standing stationary on the treadmill.

This oxygen consumption is plotted against heart rate. The line is extrapolated to the volunteer's predicted maximum heart rate, based on their age. The predicted VO_{2MAX} is the used to calculate their half maximum value for the tests on subsequent days.

No warm is thought necessary. The test increases in intensity progressively. The intensity of the test is moderate at best and the volunteer is slowly returned to rest as the belt is slowed over the next two minutes.

The volunteer is then invited for two more days: a rest and an exercise day. These are in random order. Each visit will last for 6 to 8 hours.

For the rest day, the subject visit the lab for period of about 6 hours and catheter will be inserted into subcutaneous fat and another one into the antecubital vein for collection of interstitial and blood samples respectively every 30 minutes. The microdialysis sample volume is tiny, less than 1 ml in total. The total blood sample will be less than 100 ml, comprising 10 samples of 10 ml each.
The volunteer arrives fasted. They rest for the whole period.

After about two weeks the subject will be invited again to the lab for exercise day. This lasts for a similar period. The same protocol is followed for the first 3 hours. The volunteer then exercises for 1 hour at about 50% of their maximum capacity by walking on a treadmill. During the exercise the volunteer's heart rate is measured and expired gas samples are taken at intervals. They then rest for a further 2.5 hours.

The volunteer is able to stop at any time.

5. What in your opinion are the ethical considerations involved in this proposal? (You may wish for example to comment on issues to do with consent, confidentiality, risk to subjects, etc.)
anonymity

In our opinion the ethical considerations are minor. We think that each volunteer is familiarised with the procedures. They are all adults and able to make their views clear. They are able to stop the experiment at any time.

There are very minor risks of local infection at the site of catheter insertion. All standard aseptic precautions will be taken to avoid any infections. These will include cleaning the insertion site using alcohol Wipes - 70% isopropyl alcohol, disposable single use needles, catheters gloves etc.

No significant additional risks are associated with the sub-maximal exercise intensities. The volunteers are screened with a questionnaire to confirm they are healthy before test begins. The walk up at moderate to brisk speeds. It is possible that they could stumble during this walk. Analysis of the accident records in the building reveals that slips and falls are far more common on the external steps than they are inside the building. It is concluded that the test poses no measureable increase in risk.

A first aid box available for any minor injuries might happen.

All data files will be anonymised.

The procedures may be uncomfortable at times but present no special risks.

6. Outline the reasons which lead you to be satisfied that the possible benefits to be gained from the project justify any risks or discomforts involved.

The risk of minor infection at the catheter entry sites is small and standard precautions are in place to keep this at a minimum. The risks associated with sub maximal exercise test in a young healthy population are also very small. The discomfort at the site of catheter insertion is minor and short lasting. The diameters of the catheters are small. The microdialysis catheter is 0.6 millimetres in diameter and it is introduced through a sterile tube a little more than 1 millimetre in diameter. In clinical use, these catheters are left in place for up to 7 days. One applicant (RHB) has had these catheters inserted and after the initial 'sting' of insertion did not notice any uncomfortable sensation for the following 5 days
The experimenters believe these risks and discomforts to be very small. The potential benefits are large. The experiment will give information on the effects of exercise on body fat in young women. Ultimately, this may help reduce the risks of chronic diseases such as diabetes.

7. Who are the investigators (including assistants) who will conduct the research and what are their qualifications and experience? Is it necessary for the investigators to have clearance from Disclosure (Scotland) and if so, has this been completed?
Mabroukah Alzwayi is a lecturer at Sebha University, Libya. She is presently a PhD student at Glasgow University. She has worked for about ten years in clinical laboratories. She has more than ten years experience in collecting blood samples, different age groups, (children, adult and old people) and both genders. These blood samples were collected from antecubital vein. During the project she will be trained on microdialysis collection.
Dr Ronald Baxendale has extensive experience in exercise testing normal and clinical populations. He has used the micodialysis technique in experiments performed in University of Brighton, Brunel University and Oxford Brookes University.

The volunteers are all adults and Disclosure clearance is not necessary

8. Are arrangements for the provision of clinical facilities to handle emergencies necessary? If so, briefly describe the arrangements made.

All exercise testing will be sub-maximal. Only healthy young volunteers will be tested.
No special arrangements are required. We believe that standard laboratory practice is sufficient to test a group of healthy young volunteers. Standard first aid facilities are available and there is a telephone to call for help if necessary.

9. In cases where subjects will be identified from information held by another party (for example, a doctor or hospital) describe the arrangements you intend to make to gain access to this information including, where appropriate, which Multi Centre Research Ethics Committee or Local Research Ethics Committee will be applied to.

N/A

10. Specify whether subjects will include students or others in a dependent relationship and where possible avoid recruiting students who might feel to be or be construed to be under an obligation to volunteer for a project. This is most likely to be where a student is enrolled on a course where the investigator is a teacher. In

these circumstances the recruitment could be carried out by one of the other investigators or a suitably qualified third party.

Students are not in a dependent relation ship with the researchers.
Students will be recruited, if they are agreeing to participate. Such student could come from any part of the University. Glasgow University has more than 15,000 undergraduate students, 4,900 postgraduate students and 5,000 adult learners. Dr Baxendale does teach a number of courses in FBLS and Medical Schools. These courses do not make up more than 5% of the student population and so it is unlikely, but not impossible, that the volunteers will come form those courses. In these cases Mabroukah will recruit the volunteers following the standard procedures.

11. Specify whether the research will include children or people with mental illness, disability or handicap. If so, please explain the necessity of involving these individuals as research subjects.

The experiment will not include children or people with mental illness, disability or handicap.

12. Will payment or any other incentive, such as a gift or free services, be made to any research subject? If so, please specify and state the level of payment to be made and/or the source of the funds/gift/free service to be used. Please explain the justification for offering payment or other incentive.

Yes. We propose to repay travel expenses to volunteers. They will be asked to provide receipts and repayment will be calculated following the Glasgow University guidelines up to a maximum of £20. Given the time commitment required, it may be necessary to offer some financial reward to help with recruitment. A maximum payment of £100 to each volunteer is proposed. This will come from the bench fees associated with the project.

13. Please give details of how consent is to be obtained. A copy of the proposed consent form, along with a separate information sheet, written in simple, non-technical language MUST ACCOMPANY THIS PROPOSAL FORM.

The subjects will be invited to discuss the experiment and any questions will be answered, also written information sheet will be given to them, and they are free to stop the test at any time they want.
They will be invited to sign a consent form.

14. Comment on any cultural, social or gender-based characteristics of the subject which have affected the design of the project or which may affect its conduct.

The study aims to investigate the metabolic responses of healthy young women. No unhealthy people will be recruited. No men will be recruited. This is not bias, it is simply a question of experimental design.
We think there are no cultural or social based characteristics of the volunteer population that may affect the design of the project.

15. Please state who will have access to the data and what measures which will be adopted to maintain the confidentiality of the research subject and to comply with data protection requirements e.g. will the data be anonymised?
Only the experimenters will have access to the data.
These data will be recorded using anonymised serial numbers to prevent identification of the volunteers.

16. Will the intended group of research subjects, to your knowledge, be involved in other research? If so, please justify.

It is the aim of the experimenters to avoid recruiting volunteers who are participating on other concurrent experiments.
Given that, Glasgow University has more than 15,000 undergraduate students, 4,900 postgraduate students and 5,000 adult learners and many of these will be in courses with experimental components, it is impossible to estimate how many students might be involved in one or more experiments. The applicants do not have any means of listing all the current projects in the University. The University has 6,000 staff. If the staff only runs one project each the possible combination must be astronomically high!

Those who are participating in exercise or diet restricted studies will be excluded.

17. Date on which the project will begin 1st March 2010.and end..1st March 2011..

18. Please state location(s) where the project will be carried out.

Exercise science laboratories on level 2 of the West Medical Building

19. Please state briefly any precautions being taken to protect the health and safety of researchers and others associated with the project (as distinct from the research subjects) e.g. where blood samples are being taken

Standard aseptic techniques will be used to handle fluid samples.
No other additional health or safety risk has been specially identified.
The researchers will be chaperoned appropriately during the anthropometry stages of the experiment.

Signed _____ Date _____
(Proposer of research)

Where the proposal is from a student, the Supervisor is asked to certify the accuracy of the above account.

Signed _____ Date _____
(Supervisor of student)

Email the completed form to: S.Morrison@bio.gla.ac.uk

And send the signed hard copy to:

Stuart Morrison
Faculty Research Office
Faculty of Biomedical & Life Sciences
West Medical Building
University of Glasgow
Gilmorehill
Glasgow
G12 8QQ

Appendix 2

A- Information sheet for the first study

An investigation of the effects of short bouts of exercise on the interstitial adiponectin concentrations in young healthy females.

You are invited to take part in a research study to investigate the effects of short bouts of exercise on adiponectin hormone secreted by body fat cells. The information on this sheet will help you understand what will happen.
Please read it carefully and I will be happy to answer any questions you have. It is very important that understand what you are going to do before you consider giving consent.

What is the aim of the study?
The aim of this study is to investigate how exercise affects adiponectin and ultimately what effect this may have on health. This may help reduce the risks of chronic diseases like diabetes.

Do I have to take part?
No, it is up to you. You are a volunteer, and you can stop when you want.

Why I have been chosen?
Because you are healthy young women within the age range under investigation.

What will happen to me?
You will be invited for three visits.

First visit:
This will last for about one hour. During this any questions will be answered. Your weight, height and body fat will be measured. Your fitness will be measured as you exercise at mild and moderate intensities by walking on a treadmill. This will involve breathing through a mouthpiece for a few minutes. You will wear a device on a belt round your chest to record your heart rate. You will walk on a treadmill for a total of 10-15 minutes at slow, moderate and brisk speeds.

Second visit:

Your second visit will last for 6 hours. This day will be either a rest day or an exercise day. For the last two days you have to come to the lab in a fasting state

On the first day, A needle, of about 1 mm diameter will be inserted through the skin of your abdomen about 40 mm left of your navel. This will be removed after a few minutes when a very fine sample tube has been introduced into the fat below the skin. Tiny volumes of fluid round the fat cells will be sampled during the experiment. You will rest for the whole period.

Third Visit:

The third day is similar to the second day, except you are invited to exercise by walking at a moderate speed on a treadmill for 1 hour. The fluid samples will be repeated.

What are the risks or side effects of taking part?

There are no special side effects or risks to taking part in this study. Similar studies have been done before.

The exercise intensities are delivered by walking at slow moderate of brisk speeds. This is extremely unlikely to cause any problems in a healthy young population.

The staffs are trained in taking the samples under sterile conditions.

There is risk of minor skin reddening at the site where the needles are inserted. This will be temporary. Some people may feel faint when they see a needle. Volunteer sit or lie flat whilst the needles are inserted and so this is unlikely to be a problem.

First aid and emergency procedures are in place for any accident.

What are the possible advantages of taking part?

There are no major advantages for volunteers. They will learn a little about their body composition and fitness. On the other hand, this type of research might improve understanding of common chronic diseases such as diabetes.

Will my taking part in this research be kept confidential?
Yes.

What will happen to the results of the research study?
The results of the study will be published in PhD thesis. They might also appear in scientific papers in a form which protects your identity. We can give you your results if you want.

Contact Details
If you have any questions please contact
Dr Ronald Baxendale, West Medical Building, the University, Glasgow. G12 8QQ
Telephone 0141 330 5344

B- Information Sheet for the second study

Plasma and interstitial adiponectin concentration on two days microdialysis catheter insertion.

You are invited to take part in a research study to examine the effects of two days microdialysis catheter insertion on adiponectin concentration in blood and subcutaneous tissue. The information on this sheet will help you understand what will happen.
Please read it carefully and I will be happy to answer any questions you have. It is very important that understand what you are going to do before you consider giving consent.

What is the aim of the study?
The aim of this study is to follow the response of adiponectin concentration in your adipose tissue to microdialysis catheter inserted for two days, and compare these concentrations with adiponectin concentration in your blood.

Do I have to take part?
No, it is up to you. You are a volunteer, and you can stop when you want.

Why I have been chosen?
Because you are healthy young person within the age range under investigation.

What will happen to me?
You will be invited for two visits (one of them should be fasting and for all period of collection). Each visit will last for about five hours. A needle, of about 1 mm diameter will be inserted through the skin of your abdomen about 40mm left of your navel. This will be removed after a few minutes when a very fine sample tube has been introduced into the fat below the skin and will left for two days unless it is not functioning. Tiny volumes of fluid round the fat cells will be sampled during the experiment. In addition, blood catheter will insert into cubital vein for blood collection. Dialysat and 2 ml blood will collect every hour for total five samples every day.
These catheters are very fine and you could do your usual work as usual.

What are the risks or side effects of taking part?

There are no special side effects or risks to taking part in this study. Similar studies have been done before.

The staffs are trained in taking the samples under sterile conditions.

There is a minor skin reddening at the site where the needles are inserted. This will be temporary. Some people may feel faint when they see a needle. Volunteer sit or lie flat whilst the needles are inserted and so this is unlikely to be a problem.

First aid and emergency procedures are in place for any accident.

What are the possible advantages of taking part?

There are no major advantages for volunteers. They will learn a little about their body composition.

Will my taking part in this research be kept confidential?

Yes.

What will happen to the results of the research study?

The results of the study will be published in PhD thesis. They might also appear in scientific papers in a form which protects your identity. We can give you your results if you want.

Contact Details

If you have any questions please contact

Dr Ronald Baxendale, West Medical Building,

The University, Glasgow. G12 8QQ

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West Medical Building, Faculty of Life Sciences, Glasgow University

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C- Information Sheet for the third study

Concentration of plasma high molecular weight adiponectin following acute aerobic exercise in normal women.

You are invited to take part in a research study to investigate the effects of short bouts of exercise on some hormones in plasma. These are high molecular weight adiponectin, insulin, tumour necrosis factor alpha (TNF-α) and interleukin- 6 (IL-6). The information on this sheet will help you understand what will happen.
Please read it carefully and I will be happy to answer any questions you have. It is very important that understand what you are going to do before you consider giving consent.

What is the aim of the study?
The aim of this study is to investigate how exercise affects these hormones and ultimately what effect these may have on health. This may help reduce the risks of chronic diseases like diabetes.

Inclusion criteria,
You are healthy young women. You will between 20 and 30 years old. You will have no special history of athletic training and you will not be overweight.

Do I have to take part?
No, it is up to you. You are a volunteer, and you can stop when you want.

What will happen to me?
You will be invited for four visits.

First visit.
This will last for about 30 minutes. During this any questions will be answered. Your weight, height and body fat will be measured. Your fitness will be measured as you exercise at mild and moderate intensities by walking on a treadmill. This will involve breathing through a mouthpiece for a few minutes. You will wear a device on a belt round your chest to record your

heart rate. You will walk on a treadmill for a total of 10-15 minutes at slow, moderate and brisk speeds.

Second visit

Your second visit will last for 2 hours. This day will be either a rest day or an exercise day. For these two days you have to come to the lab in a fasting state

On the rest day, blood cannula will be inserted into your vein (the needle will be removed after a few minutes when a very fine sample tube has been introduced into the vein) to collect 8 ml of blood every hour, 3 samples (0, 1, 2). (24 ml of blood totally will be collected on that day). You will rest for the whole period.

Third Visit

The third day is similar to the second day, except you are invited to exercise by walking at a moderate speed on a treadmill for 1 hour. The blood samples will be repeated.

Fourth visit

This visit will last only for 10 minutes and you will come for this visit twice one after resting day and one after exercise day, by 2 days (about 48 hours) just to collect a sample of blood (8 ml) using small fine needle.

What are the risks or side effects of taking part?

There are no special side effects or risks to taking part in this study. Similar studies have been done before.

The exercise intensities are delivered by walking at slow moderate of brisk speeds. This is extremely unlikely to cause any problems in a healthy young population.

The staffs are trained in taking the samples under sterile conditions.

There is risk of minor skin reddening at the site where the needles are inserted. This will be temporary. Some people may feel faint when they see a needle. Volunteer sit or lie flat whilst the needles are inserted and so this is unlikely to be a problem.

First aid and emergency procedures are in place for any accident.

What are the possible advantages of taking part?

There are no major advantages for volunteers. They will learn a little about their body composition and fitness. On the other hand, this type of research might improve understanding of common chronic diseases such as diabetes.

£100 will be paid for each subject at the end of the experiment.

Will my taking part in this research be kept confidential?

Yes.

What will happen to the results of the research study?

The results of the study will be published in PhD thesis. They might also appear in scientific papers in a form which protects your identity. We can give you your results if you want.

Contact Details

If you have any questions please contact

Dr Ronald Baxendale, West Medical Building, The University, Glasgow. G12 8QQ

Telephone 0141 330 5344

Mabroukah M. Alzwayi

West Medical Building, School of Life Sciences, Glasgow University

Mobile 07786694228

Email, m.alzwayi.1@research.gla.ac.uk

Appendix 3

A- Consent form

Ethics Committee for Non Clinical Research Involving Human Subjects, Material or Data

Consent Form

Title of Project: An investigation of the effects of short bouts of exercise on adiponectin in young healthy females.

Name of Researcher: Mabroukah M.A. Alzwayi.

Please initial boxes

I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and with no consequences ☐

I agree for my samples to be used for future research YES/NO (delete as appropriate) ☐

I AGREE TO TAKE PART IN THE ABOVE STUDY.

Name of Volunteer: _____

Signature: _____ Date: _____

Name of person taking consent (if different from researcher): _____

Signature: _____ Date: _____

Name of Researcher: _____

Signature: _____ Date: _____

Witness: _____ Date: _____

B- Volunteer questionnaire for short bouts exercise

Name _____ age:_____years
Height _____metre. Weight_____ Kg VO_{2max} _____
Total body fat percentage: _____ Total skin fold:_____

You are excluded from the study if you feel unwell on the day of exercise or if you are pregnant or likely to be pregnant. Furthermore if you are diabetic or one of your first relatives is diabetic.

1- Do you have high or low blood pressure? Yes/No
2- Do you have a heart condition? Yes /No
3- Have you ever felt pain in your chest when you do physical exercise? Yes / No
4- Is your doctor currently prescribing you drugs or medications? Yes/No
5- Have you ever suffered from shortness of breath at rest or with mild exercise? Yes /No
6- Is there a history of sudden death in people under 40 in your family? Yes /No
7- Do you currently smoke? Yes / No
8- Can you think of any other reason why you should not take part in our experiment? Yes /No

Please specify_____

I have read, understand and completed this questionnaire.
Name: - _____. Signature: _____ Date: _____

Appendix 4

Servomex 1440 gas analyser operating procedure:

Machine is left switched on all the times.
Approximately 30 minutes before machine is calibrated switch on both pumps using silver toggle switches on front panel.
Machine will be measuring atmospheric air and should be reading 20.9% oxygen and 0.003% carbon dioxide.
Open both cylinders, Oxygen Free Nitrogen and mixed O_2 & CO_2.
Gently open the flow meter on the OFN cylinder so that the float just moves. It is important not to increase the flow above this level.
Leave until the machine gives a stable reading of zero and adjust the ZERO control with screwdriver if required.
Turn off flow meter and check that the reading returns to atmospheric values.
Repeat using the mixed calibration gas and the machine should show 16% O_2 and 6% CO_2. Adjust SPAN with screwdriver if required.
Turn off valve and check the values return to atmospheric levels.

Ensure both gas cylinders are closed.

Once test is finished turn off pumps but do not switch off machine

Appendix 5

Oxygen consumption calculation

Oxygen uptake and carbon dioxide production at rest

1. Evacuate a Douglas bag and fit connecting tubing, a valve box and a mouthpiece.
2. Ensure that your subject is sitting quietly for 5-10 minutes before any measurements are made.
3. Place the mouthpiece in the subject's mouth and fit the nose-clip. Allow the subject to breathe quietly through the mouthpiece out to the room for 2-3 minutes before collecting air into the Douglas bag
4. Take a six-minute expired air sample.
5. Withdraw a sample of air from the Douglas bag to measure the percentage of oxygen and carbon dioxide in the gas using the paramagnetic oxygen analyser and infra-red carbon dioxide analyser.
6. Measure the volume of air in the bag using the dry gas meter. Record the temperature of the gas during evacuation. Take a note of the barometric pressure.
7. Divide the volume by six to calculate the volume of air expired per minute.
8. Convert the gas volume to standard temperature and pressure for a dry gas (STPD). See below.

Standardising Gas Volumes

Gas volumes vary with temperature and pressure. Thus, to compare respiratory gas volumes on different days and with different ambient conditions, it is necessary to correct volumes to standard conditions. Standard pressure is defined as 760 mm Hg and standard temperature as 273

K (0°C). Gas volumes are measured under ambient conditions, i.e. the conditions at the time of the experiment, and subsequently converted to STPD using the gas laws.

The universal gas equation:

$$\mathbf{PV/T = constant} \quad \text{OR} \quad \mathbf{P_1V_1/T_1 = P_2V_2/T_2}$$

Where P is pressure, T is temperature and V is volume.

As expired air is saturated with water vapour, an additional correction factor is required to correct volumes to standard conditions. Water vapour exerts a pressure which reduces the gas pressure by an amount related to temperature.

$$\text{So,} \quad \mathbf{\dot{V}E_{STPD} = \dot{V}E_{ATPS} \times (BP - SWVP)/760 \times 273/ (273 + t)}$$

Where: BP is barometric pressure in mm Hg

SWVP[#] is saturated water vapour pressure in mm Hg at ambient* temperature

t is ambient temperature in degrees Celsius

ATPS is ambient temperature and pressure, saturated with water vapour.

*Ambient temperature and pressure describe the conditions as the volume of expired air sample is measured (not necessarily the same as room temperature).

[#]SWVP = (1.1001 x t) – 4.19

Where t is temperature in degrees Celsius and SWVP is in mm Hg

9. Calculate oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) rates at rest. See box over the page.

Calculation of oxygen uptake and carbon dioxide production

Oxygen uptake ($\dot{V}O_2$) = volume O_2 inspired – volume O_2 expired

Volume O_2 inspired = $\dot{V}I \times F_IO_2\%/100$

Where: $\dot{V}I$ is the inspired air volume (per minute)

$F_IO_2\%$ is the percentage of oxygen in atmospheric air, assumed to be 20.93% (this is very stable)

Volume O_2 expired = $\dot{V}E \times F_EO_2\%/100$

Where: $\dot{V}E$ is the measured expired air volume (per minute)

$F_EO_2\%$ is the measured percentage of oxygen in expired air

[Remember to use STPD gas volumes for these calculations]

Similarly,

Carbon dioxide production ($\dot{V}CO_2$) = volume of CO_2 expired – volume CO_2 inspired

Volume CO_2 expired = $\dot{V}E \times F_ECO_2\%/100$

Where: $\dot{V}E$ is the measured expired air volume (per minute)

$F_ECO_2\%$ is the measured percentage of carbon dioxide in expired air

Volume CO_2 inspired = $\dot{V}I \times F_ICO_2\%/100$

Where: $\dot{V}I$ is the inspired air volume (per minute)

$F_ICO_2\%$ is the percentage of carbon dioxide in atmospheric air, assumed to be 0.03% (this is stable, unless there are a large number of people in a confined place).

$F_IO_2\%$, $F_ICO_2\%$, $F_EO_2\%$, $F_ECO_2\%$ and $\dot{V}E$ are known (either assumed or measured) but $\dot{V}I$ is not known. While it may seem intuitively obvious that $\dot{V}I$ should equal $\dot{V}E$, this is not always true and we cannot assume it to be the case. This explained below:

The inequality between inspired and expired gas volumes

If carbohydrate is the **only** energy source then $\dot{V}I$ does equal $\dot{V}E$, as illustrated by the equation for glucose oxidation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 38\ ATP$$

One mole of any gas occupies the same volume at STPD (22.4 l) so, for every volume of oxygen used, an equal volume of carbon dioxide is produced. It therefore follows that $\dot{V}I$ equals $\dot{V}E$, in this circumstance. The ratio of carbon dioxide production ($\dot{V}CO_2$) to oxygen uptake ($\dot{V}O_2$) is called the respiratory exchange ratio (R or RER), and when 100% carbohydrate is oxidised this equals 6/6 = 1. However, carbohydrate is not the only energy substrate. In fact, at rest and during light exercise, fat is likely to be a quantitatively more important fuel. The equation for the oxidation of palmitic acid, a typical fat, is:

$$C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O + 130\ ATP$$

For every 23 moles of oxygen utilised, 16 moles of carbon dioxide are produced, thus it is clear that $\dot{V}I$ is greater than $\dot{V}E$. The RER is 16/23 = 0.7. Thus, from the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$ it is possible to calculate the relative contribution of carbohydrate and fat to energy provision (protein oxidation is negligible under normal circumstances). An RER of 0.7 denotes 100% of energy provision is from fat oxidation, an RER of 1.0 denotes 100% carbohydrate oxidation, an RER of 0.85 denotes 50% carbohydrate oxidation and 50% fat oxidation.

On the other hand, during high intensity exercise, $\dot{V}E$ is often greater than $\dot{V}I$ because carbon dioxide production exceeds oxygen uptake. This is because lactic acid accumulates in the circulation at high exercise intensities. To prevent blood pH from falling this is buffered by bicarbonate, which is one of the most important buffers in blood, as follows:

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2$$

Thus, carbon dioxide is produced as a result on buffering by bicarbonate is excreted at the lung, together with carbon dioxide produced by oxidative metabolism. This results in an excess of carbon dioxide production over oxygen uptake and $\dot{V}E$ is greater than $\dot{V}I$. (Under these circumstances it is not

possible to calculate the relative energy provision from fat and carbohydrate from the RER).

The assumption that $\dot{V}I$ equals $\dot{V}E$ is therefore invalid and it is necessary to calculate $\dot{V}I$. This can be done using the Haldane Transformation.

The Haldane Transformation relies on the fact that there is not net uptake or production of nitrogen at the lung. The mass of nitrogen inspired therefore equals the mass of nitrogen expired. Concentration is mass/volume, thus if the concentration of nitrogen differs in inspired and expired air, there must be a difference between $\dot{V}I$ and $\dot{V}E$. If $\dot{V}E$ and the concentration of nitrogen in inspired and expired air are known, $\dot{V}I$ can be deduced. The nitrogen concentration is not measured, but determined by subtracting oxygen and carbon dioxide concentrations from 100%. The calculation is described below:

Mass inspired nitrogen = mass expired nitrogen

Mass = concentration x volume

So, $F_IN_2\% \times \dot{V}I = F_EN_2\% \times \dot{V}E$

Therefore: $\mathbf{\dot{V}I = \dot{V}E \times F_EN_2\%/F_IN_2\%}$

Where: $F_IN_2\% = 100 - F_IO_2\% - F_ICO_2\%$

$F_EN_2\% = 100 - F_EO_2\% - F_ECO_2\%$

Thus, $F_IO_2\%$, $F_ICO_2\%$, $F_EO_2\%$, $F_ECO_2\%$, $\dot{V}E$ and $\dot{V}I$ are all known and $\dot{V}O_2$ and $\dot{V}CO_2$ can be calculated:

$$\dot{V}O_2 = \dot{V}I \times F_IO_2\%/100 - \dot{V}E \times F_EO_2\%/100$$

$$\dot{V}CO_2 = \dot{V}E \times F_ECO_2\%/100 - \dot{V}I \times F_ICO_2\%/100$$

Appendix 6

A- CMA 66 Microdialysis Catheter

Box 2, SE-171 18 Solna, Sweden.
Tel: +46 8 470 10 00 Fax: +46 8 470 10 50.
E-mail: cma@microdialysis.se
Web: www.microdialysis.com

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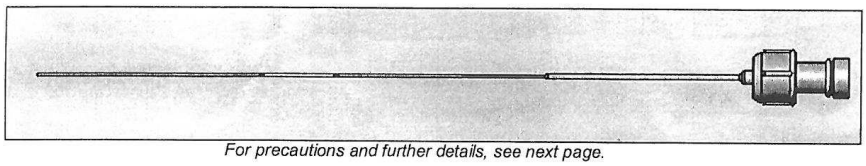
Instructions for use

CMA 66 Linear Catheter & CMA 66 High Cut Off Linear Catheter



For precautions and further details, see next page.

INTENDED USE

The ***CMA 66 Linear Catheter*** and ***CMA 66 High Cut Off Linear Catheter*** is intended to enable microdialysis in skin, adipose and resting skeletal muscle tissues.

USAGE

The procedure for insertion should be performed by a physician or a nurse under aseptic conditions. The following procedure describes insertion in skin tissue.

1. Make marks for catheter entrance and exit holes with circles (Fig 1).
2. Take the introducer cannula and insert it through the circle marking the catheter exit hole (Fig 2) and continue through the skin to the marked catheter entrance hole (Fig 3). Avoid penetration of actual markings.

Fig 1

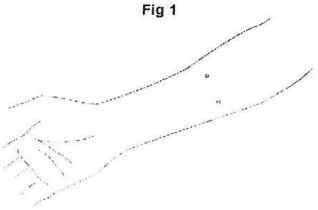


Fig 2

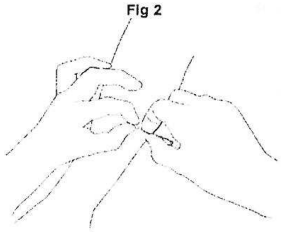
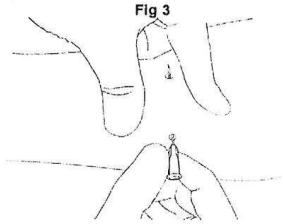


Fig 3



3. Remove the catheter from the protective tube and guide the catheter gently in to the sharp end of the introducer cannula (Fig 4). Push it ***carefully*** through the introducer cannula until it appears at the opposite side of the cannula and continue until the membrane is in position (Fig 5).
   Important: Be very gentle when entering the membrane into the introducer cannula.
4. When the catheter and membrane is in position, the introducer cannula is removed (Fig 6), after which the catheter is fixed to the skin.

Fig 4

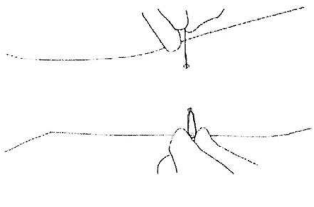


Fig 5

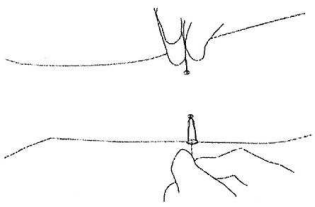
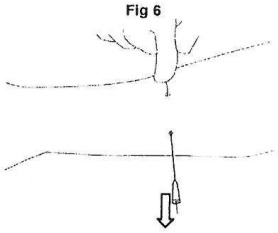


Fig 6



5. Connect the sample vial (Fig 7, 8, 9). It is important to position the vial at the same level or lower than the membrane in order to avoid ultrafiltration through the membrane.

Art. nr. 8010603D
2010-01-28

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Web: www.microdialysis.com

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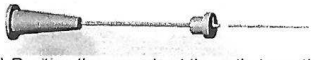
Fig 7 (a, b)

*a) Position the vial cap and a cannula (preferably **21G**).*

b) Penetrate vial cap with the cannula.

Fig 8 (a, b, c)



a) Position the cannula at the catheter outlet

b) Carefully guide the catheter in to the cannula

c) Remove the cannula from the outlet

Fig 9 (a, b)

a) Connect the 300 µl glass vial

b) Or connect the CMA Microvial

6. Connect the catheter to the syringe filled with perfusion fluid (CMA 106 Syringe) and place the syringe in the pump (CMA106 or CMA107) and close the lid. Check that fluid comes out of the outlet, and into the vial within approximately 2 minutes.

REMOVAL OF THE CATHETER

The removal of the catheter shall be performed under aseptic conditions.
Cut the outlet tube as short as possible. The catheter is then removed by carefully pulling it out of the tissue.
NOTE: Discontinued/removed catheters shall be handled according to the hospital routines for biohazard material.

CONTRAINDICATIONS & RISKS

For ***CMA 66 High Cut Off Linear Catheter***: Patients with known hypersensitivity to Dextran.

PRECAUTIONS

- Care must be taken to avoid needlestick injuries to reduce risk of exposure to contaminated blood.
- The CMA 66 Linear catheter shall only be used with the introducer cannula included in the package.
- The *CMA 66* ***High Cut Off*** *Linear Catheter* should be perfused with a mixture of Ringer and Dextran (30g Dextran 60/1000mL) to avoid ultrafiltration through the membrane.
- Remove the catheter if there are any signs of infection.
- This device is sterile unless the package has been opened or damaged.
- The CMA 66 shall only be used together with the accessories described in the table below.
- Be sure to handle the catheter carefully to avoid kinking or other damage, particularly after removal of the protection tube. **Avoid contact with the dialysis membrane.**
- If any visible damage is observed, do not use the catheter.
- If there is a suspicion that the catheter has become unsterile prior to insertion the catheter shall not be used.
- Check that liquid is being pumped through the catheter by inspecting the volume in the microvial **each time** the microvials are changed.
- Remove the catheter if there is a permanent stop in the liquid flow.

DELAY IN THE SYSTEM AND THE TIME IT TAKES TO FILL UP A MICROVIAL

Flow rate	Delay time (with 100mm outlet)	Time to acquire minimum volume for analysis in CMA analyzers	Time to fill a microvial (200 µl)
0,3 µl/min	≈ 6 min	9 – 14 min*	10 hours
0,5 µl/min	≈ 3 min	5 – 8 min*	6 hours
1 µl/min	≈ 2 min	3 – 4 min*	3 hours

*Depending on the number of substances that shall be analyzed

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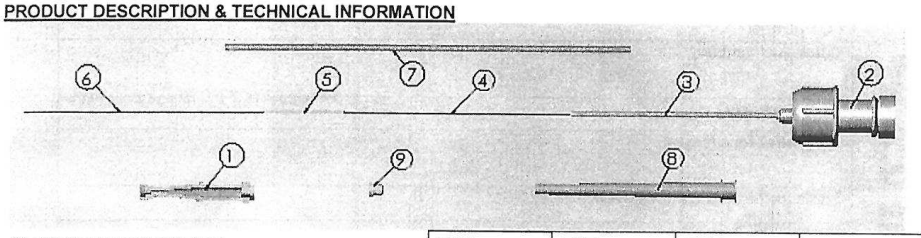
PRODUCT DESCRIPTION & TECHNICAL INFORMATION



X= Identical on all catheters	REF 8010650	REF 8010651 (High cut off)	REF 8010670	REF 8010671 (High cut off)
1. Microvial with vial cap (Polystyrene + Santoprene)	X	X	X	X
2. Luerlock-connector (Polycarbonate)	X	X	X	X
3. Inlet tube (Polyurethane, OD 1 mm)	50 mm	50 mm	50 mm	50 mm
4. Inlet tube (Polyurethane, OD 0,4 mm)	350 mm	350 mm	350 mm	350 mm
5. Membrane (Polyarylethersulphone, PAES, OD 0.5 mm)	30 mm	30 mm	10 mm	10 mm
6. Outlet tube (Polyurethane, OD 0,4 mm)	100 mm	100 mm	100 mm	100 mm
7. Protective tube (Polyethylene)	145 mm	145 mm	145 mm	145 mm
8. Introducer cannula 21G (Stainless steel))	50mm	50mm	50mm	50mm
9. Vial Cap	X	X	X	X

ACCESSORIES

CMA 66 shall only be used with the following accessories:

REF	Name
8010191	CMA 106 Syringe 20/pkg
P000001	Microvials 250/pkg
P000154	Microvials in rack, Sterile 12x4
P000003	CMA 106 Microdialysis Pump
P000127	CMA 107 Microdialysis Pump
P000034	Perfusion Fluid T1
7431007	Vial Glass 300 µl
8001788	Battery 2X3 V

For CMA 66 High Cut Off Linear Catheter:

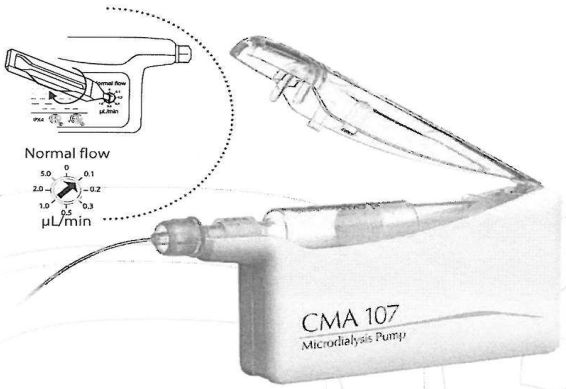
To avoid ultra filtration over the membrane the catheter should be used with a Perfusion Fluid consisting of a Ringer solution containing 30g Dextran 60/1000mL.

B- CMA107 Microdialysis Pump

CMA 107 Microdialysis Pump
a portable, adjustable flow, battery-operated pump

THE CMA 107 MICRODIALYSIS PUMP is a unique 2.5 mL syringe pump with flexibility in flow rate that enables the user to change the flow for different purposes: low flow rate for high recovery of low molecular weight substances in a tissue, or high flow rate for blood flow measurements and for more frequent sampling intervals. The operating flow is adjustable to eight different settings: 0, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0 and 5.0 µL/min.

The CMA 107 Microdialysis Pump is a portable battery-driven pump and very easy to use. The syringe is filled with 2.5 mL of sterile perfusion fluid, connected to the microdialysis catheter and then placed in the pump. When the pump lid is closed, a 5-minute flush cycle begins and is followed by an automatic decrease to the pre-set operating rate.



CAUTION: Investigational Device
Limited by United States Law to Investigational Use.
To be used only for Institutional Review Board (IRB) approved or, if applicable, FDA approved studies.

* Portable, small and lightweight * Easy to handle * Self-controlled with LED function signals
* Splash proof * ETL-listed * Variable flow

ORDERING INFORMATION

CMA 107 Microdialysis Pump

	Ref. No.
CMA 107 Microdialysis Pump	P000127

Accessories

MD Pump Kit, Peripheral Tissue	8003790
MD Pump Kit, Brain Tissue	8003791
CMA Syringe, 20/pkg	8010191
Battery, 2 x 3V	8001788
Perfusion Fluid, T1, 5 mL,10/pkg	P000034
Perfusion Fluid, CNS, 5 mL,10/pkg	P000151

TECHNICAL INFORMATION

Normal Flow: Variable, 0.1-5 µL/min, 8 settings
Flush flow: 15 µL/min
Dimension: 90 x 50 x 20 mm
Weight: 70g (incl. battery)
Battery: Lithium 6V
Casing: ABS plastic, splash proof
Operating temperature: +5 to +40°C
Alarms: Error, Low battery

C- Micro vials

Accessories
Microvials and Microvial Racks

ORDERING INFORMATION

Microvials

		Ref. No.
Microvials	250/pkg	P000001

Microvial Racks

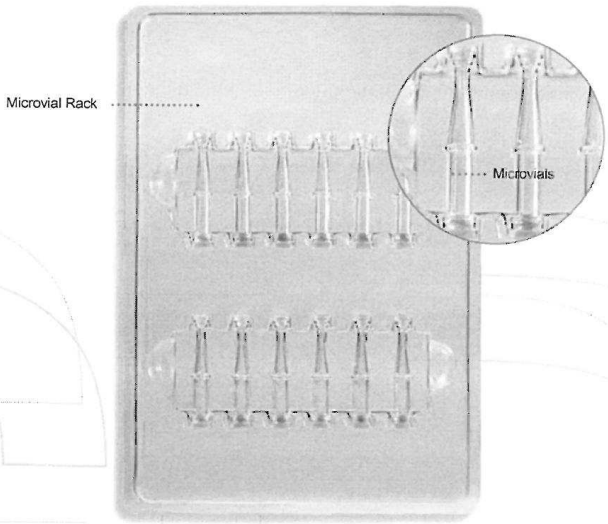
	Ref. No.
Microvial Rack, 12/pkg	P000028

Microvial Racks, Sterile

	Ref. No.
Microvials in a rack, 12 x 4	P000154

MICROVIALS are designed to collect micro-volume samples and minimize evaporation. Each vial holds 200 µL. If needed sterile microvials are packed and sold in racks for use under sterile conditions.

MICROVIAL RACKS. To minimize evaporation, samples in microvials can be placed in a Microvial Rack prior to storage in the refrigerator or freezer. The rack can store and close 12 microvials and facilitate the logistics of sample handling.



D- Perfusion fluid

MICRODIALYSIS PUMP KIT, Peripheral Tissue
This Pump Kit includes the accessories needed for single patient microdialysis monitoring in peripheral tissue. The kit contains a CMA syringe, a battery and a T1 perfusion fluid ampoule.

MICRODIALYSIS PUMP KIT, Brain Tissue
This Pump Kit includes the accessories needed for single patient microdialysis monitoring in brain tissue. The kit contains a CMA syringe, a battery and a CNS perfusion fluid ampoule.

ORDERING INFORMATION

MD Pump Kit, Peripheral Tissue

	Ref. No
MD Pump Kit, Peripheral Tissue 1 CMA 106/107 Syringe, 1 battery, 1 Perfusion Fluid T1	8003790

MD Pump Kit, Brain Tissue

MD Pump Kit, Brain Tissue 1 CMA 106/107 Syringe, 1 battery, 1 Perfusion Fluid CNS	8003791

Appendix 7

A- Mercodia adiponectin ELISA:

REAGENTS

Each Mercodia Adiponectin ELISA kit (10-1193-01) contains reagents for 96 wells, sufficient for 42 samples and one calibrator curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is 2-8℃.

Coated Plate 1 plate 96 wells Ready for use
(Mouse monoclonal anti-human adiponectin) 8-well strips
For unused microtitration strips, reseal the bag using adhesive tape and store at 2-8℃ for two months.

Calibrators 1, 2, 3, 4, 5 5 vials 1000 µl Ready for Use
(Recombinant human adiponectin)
Concentration stated on vial label. Color coded yellow

Calibrator 0 1 vial 5 ml Ready for use
Color coded yellow

Assay Buffer 1 vial 12 ml Ready for use
Color coded red

Sample Buffer 2X 1 bottle 50 ml
Dilute with 50 ml redistilled water to make sample buffer.
Color coded yellow
Storage after dilution: 2-8℃ for two months

Enzyme Conjugate 11X 1 vial 1.3 ml Preparation, see below
(Peroxidase conjugated mouse monoclonal anti-human adiponectin)

Enzyme Conjugate Buffer 1 vial 13 ml Ready for use
Color coded blue

Wash Buffer 21X 1 bottle 40 ml
Dilute with 800 ml redistilled water to make wash buffer.
Storage after dilution: 2-8℃ for two months

Substrate TMB 1 bottle 22 ml Ready for use
(TMB) Colorless solution Note! Light sensitive!
Stop Solution 1 vial 7 ml Ready for use 0.5 M H2SO4

Preparation of enzyme conjugate solution

Prepare the needed volume of enzyme conjugate solution by dilution of Enzyme Conjugate 11X,

(1+10) in Enzyme Conjugate Buffer according to the table below. Mix gently.

When preparing enzyme conjugate solution for the whole plate or if the reagents are to be used within two months, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X vial.

Storage after dilution: 2-8℃ for two months.

SPECIMEN COLLECTION AND HANDLING

Serum

Collect blood by venipuncture, allow to clot. Separate the serum by centrifugation at 4 300 g for 15 minutes at 2-8℃. Specimen can be stored at 2-8° C up to 14 days. For longer periods, store samples at –20℃. Avoid repeated freezing and thawi ng.

Plasma

Collect blood by venipuncture into tubes containing heparin, citrate or EDTA as anticoagulant, and separate the plasma fraction. Samples can be stored at 2-8℃ up to 14 days. For longer periods store samples at –20℃. Avoid repeated free zing and thawing.

PREPARATION OF SAMPLES

Samples should be diluted 1/101 v/v with sample buffer (20 µl sample + 2,0 ml sample buffer). Diluted samples can be stored at 2-8℃ up to 14 day s. Note! Buffers containing sodium azide (NaN3) can not be used for sample dilution.

TEST PROCEDURE

All reagents and samples must be brought to room temperature before use. Prepare a calibrator curve for each assay run.

1. Prepare enzyme conjugate solution (according to the table on previous page), sample buffer, wash buffer and samples.
2. Prepare sufficient microplate wells to accommodate Calibrators and samples in duplicate.
3. Pipette 25 µl each of Calibrators and samples into appropriate wells.
4. Add 100 µl of Assay Buffer into each well.
5. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18-25℃).
6. Wash plate 6 times with automatic plate washer
   Or aspirate the reaction volume completely and fill each well with 350 µl wash buffer. Aspirate liquid completely. Repeat 5 times. After final wash, invert and tap the plate firmly against absorbent paper.
7. Add 100 µl of enzyme conjugate solution into each well.
8. Incubate on a plate shaker for 1 hour at room temperature (18-25℃).
9. Wash plate 6 times with automatic plate washer

Or aspirate the reaction volume completely and fill each well with 350 µl wash buffer. Aspirate liquid completely. Repeat 5 times. After final wash, invert and tap the plate firmly against absorbent paper.

10. Add 200 µl Substrate TMB into each well.
11. Incubate for 15 minutes at room temperature (18-25℃).
12. Add 50 µl Stop Solution to each well.
    Place the plate on the shaker for approximately 5 seconds to ensure mixing.
13. Read optical density at 450 nm and calculate results.
    Read within 30 minutes.

Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.

INTERNAL QUALITY CONTROL

Commercial controls such as Mercodia Obesity Control kit (10-1241-01) and/or internal serum pools with low, intermediate and high adiponectin concentrations should routinely be assayed as unknowns, and results charted from day to day. It is good laboratory practice to record the following data for each assay: kit lot number; reconstitution dates of kit components; OD values for the blank (sample buffer), Calibrators and controls.

CALCULATION OF RESULTS

Computerized calculation

The concentration of adiponectin is obtained by computerized data reduction of the absorbance for the Calibrators 1-5 versus the concentration using cubic spline regression.

Manual calculation

1. Plot the absorbance values obtained for the Calibrators 1-5 against the adiponectin concentration on a log log paper and construct a calibrator curve.
2. Read the concentration of the unknown samples from the calibrator curve.
3. Multiply the concentration with the dilution factor.

B- Mercodia Insulin ELISA

REAGENTS 1 X 96

Each Mercodia Insulin ELISA kit (10-1113-01) contains reagents for 96 wells, sufficient for 42 samples and one calibrator curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated onthe outer label. The recommended storage temperature is 2–8℃.

Coated Plate 1 plate 96 wells Ready for Use

Mouse monoclonal anti-insulin 8-well strips

For unused microplate wells completely reseal the bag using adhesive tape and use within two months.

Calibrators 1, 2, 3, 4, 5 vials 1000 µL Ready for Use

Concentration indicated on vial label.

(recombinant human insulin) Color coded yellow

Calibrator 0 1 vial 5 mL Ready for Use

Color coded yellow

Enzyme Conjugate 11X 1 vial 1.2 mL Preparation,

Peroxidase conjugated mouse monoclonal anti-insulin see below

Enzyme Conjugate Buffer 1 vial 12 mL Ready for use

Color coded blue

Wash Buffer 21X 1 bottle 50 mL Dilute with 1000 mL

Storage after dilution: redistilled water to

2-8℃ for 4 weeks. make wash buffer 1X solution

Substrate TMB 1 bottle 22 mL Ready for Use

Colorless solution

Note! Light sensitive!

Stop Solution 1 vial 7 mL Ready for Use

0.5 M H_2SO_4

Preparation of enzyme conjugate 1X solution

Prepare the needed volume of enzyme conjugate 1X solution by dilution of Enzyme Conjugate 11X, (1+10) in Enzyme Conjugate Buffer according to the table below. When preparing enzyme conjugate 1X solution for the whole plate, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X vial. Mix gently. Use within one day.

SPECIMEN COLLECTION AND HANDLING

Serum

Collect blood by venipuncture, allow to clot, and separate the serum by centrifugation. Samples can be stored at 2–8℃ up to 24 hours. For longer p eriods, store samples at –20℃. Avoid repeated freezing and thawing.

Plasma

Collect blood by venipuncture into tubes containing heparin or EDTA as anticoagulant, and separate the plasma fraction. Samples can be stored at 2–8℃ up to 24 hours. For longer periods store samples at –20℃. Avoid repeated free zing and thawing.

Preparation of samples

No dilution is normally required, however, samples containing >200 mU/L should be diluted 1+9 v/v with Calibrator 0.

TEST PROCEDURE

All reagents and samples must be brought to room temperature before use.

Prepare a calibrator curve for each assay run.

1. Prepare enzyme conjugate 1X solution and wash buffer 1X solution.
2. Prepare sufficient microplate wells to accomodate Calibrators and samples in duplicate.
3. Pipette 25 µL each of Calibrators and samples into appropriate wells.
4. Add 100 µL of enzyme conjugate 1X solution to each well.
5. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18–25℃).
6. Wash 6 times with 700 µL wash buffer 1X solution per well using an automatic plate washer with overflow-wash function. Do not include soak step in washing procedure. Or manually, discard the reaction volume by inverting the microplate over a sink. Add 350 µL wash buffer 1X solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing.
7. Add 200 µL Substrate TMB into each well
8. Incubate for 15 minutes at room temperature (18–25℃).
9. Add 50 µL Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
10. Read optical density at 450 nm and calculate results. Read within 30 minutes.

Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.

INTERNAL QUALITY CONTROL

Commersial controls such as Mercodia Diabetes Antigen Control (Cat. No. 10-1134-01/10-1164-01) and/or internal serum pools with low, intermediate and high insulin concentration should routinely be assayed as samples, and results charted from day to day. It is good laboratory practice to record the following data for each assay: kit lot number, dilution and/or reconstitution date of components, OD values for the blank, Calibrators and Controls.

CALCULATION OF RESULTS

Computerized calculation

The concentration of insulin is obtained by computerized data reduction of the absorbance for the Calibrators, except for Calibrator 0, versus the concentration.

Appendix 8

R&D systems, Quantikine ELISA

A- Human IL-6

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

IL-6 Standard - Reconstitute the IL-6 Standard with 5.0 mL of Calibrator Diluent RD5T (*for cell culture supernate samples*) or Calibrator Diluent RD6F (*for serum/plasma samples*). This reconstitution produces a stock solution of 300 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 667 µL of the appropriate Calibrator Diluent into the 100 pg/mL tube and 500 µL of diluent into each remaining tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (300 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.
4. Add 100 µL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (Schnabele, 2009 400 /id) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of IL-6 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

B- Human TNF-α

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD6-35 (1X) (*for cell culture supernate samples*) - Add 10 mL of Calibrator Diluent RD6-35 to 40 mL of deionized or distilled water to yield 50 mL of Diluted Calibrator Diluent RD6-35.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

TNF-α Standard - **Refer to vial label for reconstitution volume.** Reconstitute the TNF-α Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-35 (*for serum/plasma samples*) or Calibrator Diluent RD6-35 (1X) (*for cell culture supernate samples*) into the 1000 pg/mL tube. Pipette 500 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1F to each well. *Assay Diluent RD1F will have a precipitate present. Mix well before and during use.*
4. Add 200 µL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (Schnabele, 2009 400 /id) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of TNF-α Conjugate to each well. Cover with a new adhesive strip.**For Cell Culture Supernate Samples:** Incubate for 1 hour at room temperature.**For Serum/Plasma Samples:** Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**

9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor

C- High molecular weight adiponectin (HMWA)

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated**.**

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 100-fold dilution. A suggested 100-fold dilution is 10 µL of sample + 990 µL of Calibrator Diluent RD6-61.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 mL of the resultant mixture is required per well.

HMW Adiponectin Standard - **Refer to the vial label for reconstitution volume.**

Reconstitute the HMW Adiponectin Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD6-61 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 250 ng/mL standard serves as the high standard. Calibrator Diluent RD6-61 serves as the zero standard (0 ng/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.

4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (Schnabele, 2009 400 /id) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of HMW Adiponectin Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the HMW Adiponectin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Appendix 9

Plasma glucose measurements

San Diego, CA 92121

GLUCOSE OXIDASE REAGENT SET

(PHENOL FREE)

Catalog Number: BQ018B-CR

INTENDED USE

For the quantitative determination of total glucose in human serum.

REAGENT COMPOSITION

When reconstituted as directed, the reagent for Glucose contains the following:

1. *Glucose reagent*: (Concentrations refer to the reconstituted reagent) Glucose Oxidase 15µl/ml, Peroxidase (horseradish) 1.2µl/ml. Mutarotase 4.0 µl/ml. 4-Aminoantipyrine 0.38 mM p-Hydroxybenzene sulfonate 10 mM, and non-reactive ingredients.
2. *Glucose standard*: (100mg/dl b-D-glucose).

WARNINGS AND PRECAUTIONS

1. for in vitro diagnostic use.

CAUTION: In vitro diagnostic reagents may be hazardous. Handle in accordance with good laboratory procedures which dictate avoiding ingestion, and eye or skin contact.

2. Specimens should be considered infectious and handled appropriately.
3. Use distilled or deionized water where indicated.

REAGENT STORAGE AND STABILITY

Both dry reagent and standard should be stored at 2 – 8 °C prior to reconstitution. The reagent may be used until the expiration date indicated on the package label. The reconstituted reagent should be stored in an AMBER container at 2 – 8 °C and is stable for thirty (30) days when stored as directed. The reagent should be clear and colorless.

REAGENT DETERIORATION

The reagent should be discarded if:

1. Turbidity has occurred; turbidity may be a sign of contamination.
2. Moisture has penetrated the vial and caking has occurred.
3. The reagent fails to meet linearity claims or fails to recover control values in the stated range.

SPECIMEN COLLECTION

1. Test specimens should be serum and free from hemolysis.
2. Plasma containing citrate, EDTA, heparin or oxalate as an anticoagulant may not be used.
3. Serum must be separated from the clot promptly since the rate of glucose decrease is approximately 7% per hour in whole blood.
4. Glucose in serum or plasma is stable for twenty-four (24) hours when stored 2 – 8 ° C.

INTERFERING SUBSTANCES

Grossly lipemic or icteric sera will cause false glucose values and require the use of a serum blank. Add 0.02 ml (20 µl) of patient sera to 3.0 ml distilled water and read against water blank. Subtract this absorbance from the patient test absorbance to correct for the lipemia or icterus. Young et al. give a comprehensive review of drug interferences.5

MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes to accurately measure required volumes
2. Test tubes/rack
3. Timer
4. 37° C heating block or water bath
5. Spectrophotometer capable of accurately measuring absorbance at 500 nm

GENERAL INSTRUCTIONS

The reagent for glucose is intended for use either as an automated procedure on chemistry instruments or as a manual procedure on a suitable spectrophotometer.

AUTOMATED PROCEDURE

Please refer to appropriate application manual available.

MANUAL PROCEDURE

1. Prepare reagent according to instructions.
2. Label test tubes: blank, standard, control, patient, etc.
3. Pipette 1.5 ml of working reagent to all tubes and place in 37 °C heating bath for at least five (5) minutes.
4. Add 0.01 ml (10 µl) of sample to respective tubes, mix and incubate at 37 °C for exactly ten (10) minutes.
5. After incubation, zero spectrophotometer with the reagent blank. Read and record the absorbance of all tubes at 500 nm (Wavelength range: 500 – 520 nm). Final colour is stable for at least thirty (30) minutes.

** TC MULTI-PURPOSE CALIBRATOR MAY BE USED TO*
REPLACE STANDARD.

NOTE:

If the spectrophotometer being used requires a final volume greater than 1.5 ml for accurate reading, use 0.02 ml (20 µl) of sample to 3.0 ml of reagent. Perform the test as described above.

LIMITATIONS

The reagent is linear to 500 mg/dl. Samples that have glucose values greater than 500 mg/dl should be diluted with water 1:1.