THE NATURE OF CACHEXIA IN PATIENTS WITH HEART FAILURE AND STABLE CORONARY ARTERY DISEASE

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Submitted October 2007 in fulfilment of the degree Doctor of Philosophy to the Faculty of Biomedical & Life Sciences, University of Glasgow, United Kingdom

Declaration

I hereby declare that this thesis was composed by myself and that all the work reported was performed by myself except where assistance has been acknowledged. This work has not previously been submitted for a higher degree. All sources of information have been acknowledged by reference.

Some of the results contained in this thesis have previously been published and presented:

McEntegart MB, Awede B, Petrie MC, Sattar N, Dunn FG, MacFarlane NG, McMurray JJV. Increase in serum adiponectin concentration in patients with heart failure and cachexia: relationship with leptin, other cytokines and B-type natriuretic peptide. Eur Heart J. 2007; 28: 829-35.

McEntegart MB, Sattar N, Awede B, Petrie MC, Dunn FG, MacFarlane NG, McMurray JJV. Increase in serum adiponectin concentration in patients with heart failure and cachexia – cause or consequence? British Heart Foundation Cardiovascular Research Centre Symposium. University of Glasgow. 2006.

M.B.McEntegart

Acknowledgements

I would like to start by thanking my supervisor Dr Niall MacFarlane. Niall conceived and proposed the original hypothesis for this project and has guided and supported me throughout my doctoral work. Thank you Niall for your commitment, encouragement and friendship over the last few years.

I would also like to thank my co-supervisor Professor John McMurray for providing me with this opportunity to pursue doctoral training. With his drive and pursuit of excellence he has enabled me to gain a wide an invaluable experience of academic cardiology during this period of study.

I am indepted to Niall and Professor McMurray for their efforts in helping me secure a British Heart Foundation Research Fellowship to finance this project, and for somehow finding a little extra when it was needed.

Dr Frank Dunn, through his recommendation, opened the door for me to this opportunity. I would like to thank him for this and for his constant encouragement and steady support over the years.

I wish to thank Dr Bonaventure Awede for patiently helping me brush up my bench skills and for being a constant source of practical advice throughout my doctoral training.

I am very grateful to Professor Mina Behan who shared her expertise and enthusiasm with me despite her deteriorating health during this project. Professor Behan sadly died shortly after this work was completed. She is very fondly remembered by myself and the many other students fortunate to benefit from her guidance.

Further thanks to Dr Mark Petrie for his help with patient recruitment, and Professor Naveed Sattar for his collaboration.

Thanks to Barbara, Lynne, Karen and Joan, the staff at the Cardiovascular Investigations and Research Unit at the Western Infirmary, for all their help with the patient visits, and for the coffee and the chat.

Most importantly, I would like to thank the patients and volunteers who generously gave their time and without whom this research could not have been done. Some of these patients were significantly debilitated and yet some how found the motivation to participate. Each remains firmly fixed in my memory of this important time in my training.

Contents

Summary

Cachexia is a prognostically important development in patients with heart failure. The most commonly used definition of cardiac cachexia is loss of a percentage of body weight over time. Muscle wasting has been assumed to be the major contributor to this weight loss, and cytokine activation is postulated to be central to the pathogenesis. We hypothesised that elevated circulating cytokines in cachectic heart failure patients would be associated with muscle inflammation, injury and impaired ability to repair.

The aim of this doctoral work was to characterise the nature of cachexia in patients with heart failure (HF) and stable coronary artery disease (CAD), to quantify the loss of muscle mass, and test the hypothesis that muscle wasting is mediated by the activation of tissue cytokines and cell cycle inhibitors.

We studied five subject groups. Three were groups of patients with stable coronary artery disease: 1) HF-cachexia - patients with HF, reduced left ventricular systolic function and cachexia, n=10; 2) HF-no cachexia - those with HF, reduced systolic function but no cachexia, n=20; and 3) CAD - those with CAD, no symptoms of HF and preserved systolic function, n=10. The other subject groups were: 4) IDCM – patients with idiopathic dilated cardiomyopathy, n=7; and 5) HC – healthy controls, n=9.

Subjects were characterised by New York Heart Association (NYHA) classification, left ventricular ejection fraction (LVEF), peak oxygen consumption (VO_2), weight

history and body composition analysis. Circulating levels of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), leptin, adiponectin, and B-type natriuretic peptide (BNP) were measured. Skeletal muscle biopsies were analysed for the expression of messenger ribonucleic acid (mRNA) for TNF-α, IL-6, interleukin-1β (IL-1β), interleukin-18 (IL-18) and the cell cycle inhibitors (cyclin dependent kinase (CDK) inhibitors) p21, p27 and p57.

We found that the HF-cachexia group had significantly lower body mass index (BMI) and percentage body fat than all the other subject groups. In contrast, there was no significant reduction in fat free mass index (FFMI). In addition, the HF-cachexia group had higher rates of fat oxidation than all other groups.

While the HF-cachexia group had elevated circulating levels of TNF-α and IL-6, there was no increased expression of cytokines or CDK inhibitors in the skeletal muscle.

Circulating adiponectin and BNP levels were elevated in the HF-cachexia group. There was a positive association between adiponectin and BNP, and a negative relationship of each with BMI and percentage body fat. In addition, adiponectin positively correlated with rate of fat oxidation and TNF-α concentration.

A possible causal relationship between adiponectin and increased rate of fat oxidation was further investigated in an additional study of young healthy male subjects performing an exercise program specifically designed to maximise fat metabolism

(n=11). Despite inducing significantly increased rates of fat oxidation and adiponectin concentrations no relationship was observed between them.

In conclusion, cachexia in patients with heart failure and stable coronary artery disease predominantly involves the loss of adipose tissue, with no evidence of muscle wasting or inflammation. The presence of increased circulating levels of adiponectin and BNP, their association with each other, and the relationship of each with body composition, energy metabolism and TNF-α suggests these peptides may play an important role in the pathogenesis of cardiac cachexia.

List of Abbreviations

A	Adenine
ACC	American College of Cardiology
ACE	Angiotensin converting enzyme
AHA	American Heart Association
ANOVA	Analysis of variance
ARB	Angiotensin receptor blocker
BIA	Bioelectrical impedance analyser
BMI	Body mass index
BNP	B-type natriuretic peptide
C	Cytosine
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
CHF	Chronic heart failure
CHO	Carbohydrate
Cl^{-1}	Chloride
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CYC	Cytochrome C
dH_2O	Distilled water

DNA	Deoxyribonucleic acid
DEXA	Dual x-ray absorbitometry
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
ESC	European Society of Cardiology
FFMI	Fat free mass index
G	Guanine
H^+	Hydrogen
Hb	Haemoglobin
HDL	High density lipoprotein
HF	Heart failure
H_2O	Water
HOMA1-IR	Homeostasis model assessment index
IDCM	Idiopathic dilated cardiomyopathy
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-18	Interleukin-18
K^+	Potassium
LDL	Low density lipoprotein
LVEF	Left ventricular ejection fraction
MDRD	Modification of diet in renal disease
Mg^{2+}	Magnesium

MRF	Muscle regulatory factor
mRNA	messenger ribonucleic acid
Na^+	Sodium
NT-proBNP	N-terminal pro B-type natriuretic peptide
NYHA	New York Heart Association
O_2	Oxygen
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
RTPCR	Real time polymerase chain reaction
SD	Standard deviation
SEM	Standard error on the mean
T	Thymine
TBW	Total body water
TNF-α	Tumour necrosis factor alpha
VLDL	Very low density lipoprotein
VO_2	Oxygen consumption
VCO_2	Carbon dioxide production

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CHAPTER 1

GENERAL INTRODUCTION

Introduction

Cachexia is the wasting process associated with malignancy and chronic disease, involving the loss of skeletal muscle, adipose tissue and bone. Although cachexia has been identified as a prognostically important development in heart failure, its definition, aetiology and pathophysiology remain the subject of debate (Anker *et al*, 1997; Von Haehling *et* al, 2007).

Muscle wasting has commonly been assumed to be the major contributor to weight loss in cardiac cachexia. While cytokine activation is one of the most frequently postulated causes of cachexia in heart failure, ageing, muscular dystrophy, and other chronic diseases are known to be associated with activation of cell cycle inhibitors, impairing proliferation of muscle progenitor cells and muscle repair. (Levine *et al*, 1990; McMurray *et al*, 1991; Dutka *et al,* 1993; Anker *et al,* 1997; Welle *et al,* 2004; Endesfelder *et al,* 2000; Dasarathy *et al,* 2004; Fulds, 2005).

More recently, it has been suggested that the loss of body fat is of prognostic importance in heart failure, stimulating interest in adipose tissue derived cytokines and hormones, and the regulation of energy metabolism (Lavie *et al,* 2003).

Heart Failure

Definition of heart failure

Heart failure is a common, costly, disabling and deadly disease with increasing prevalence. Heart Failure has been defined by both the European Society of Cardiology (ESC) Task Force and the American College of Cardiology/American Heart Association (ACC/AHA) (Swedberg *et al*, 2005; Hunt *et al*, 2005). The ESC guidelines define heart failure as 'a syndrome where the patients should have the following features; symptoms of heart failure, typically breathlessness or fatigue, either at rest or during exertion, or ankle swelling, and objective evidence of cardiac dysfunction at rest.' Similarly, the ACC/AHA guidelines define heart failure as a 'clinical syndrome that is characterised by specific symptoms (dyspnoea and fatigue) and signs (fluid retention)' and states that 'there is no diagnostic test for heart failure, because it is largely a clinical diagnosis that is based on a careful history and physical examination.' It continues 'Heart failure is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood'.

Epidemiology of heart failure

The combination of improved survival post myocardial infarction and the ageing population has lead to an increase in the prevalence of heart failure. In Scotland in the year 2000 the prevalence of heart failure was 7.1 in 1000, increasing with age to 90.1 in 1000 in patients >85 years (Murphy *et al*, 2004). By 1999, in the USA, hospitalisations with heart failure had increased to over 1 million per annum (Koelling *et al*, 2004).

The cost of managing heart failure in the early 1990s was estimated to be 1–2% of total health care expenditure in industrialised countries, with this likely to be a considerable underestimate of current costs with increased prevalence and hospitalisation rates through the 1990s (McMurray *et al*, 1998).

The Framingham study reported a mortality rate of approximately 60% within five years of a diagnosis of heart failure (Ho *et al*, 1993). In Scotland in 1991 patients admitted to hospital with a first presentation of heart failure had a five-year survival of only 25%. With the exception of lung cancer, survival with heart failure was worse than with all the other most common cancers and with myocardial infarction (Stewart *et al*, 2001).

Aetiology of heart failure

In western countries coronary artery disease followed by hypertension and dilated cardiomyopathy are the most common causes of heart failure. In 1965 the Framingham study found hypertension to be the most common cause, being the primary cause in 30% of men and 20% of women. During long-term follow up the prevalence of coronary artery disease increased in the population and became the primary cause of heart failure in 70% of cases in the 1970s. At the same time the relative contribution of hypertension and valvular heart disease declined (Kannel *et al*, 1994). This is also reflected in more recent large clinical trials and registry populations (McMurray *et al*, 2000). A Glasgow study of left ventricular systolic dysfunction found that, in patients who also had symptoms of heart failure, 95% had

evidence of coronary artery disease, 80% hypertension and 25% valvular heart disease (McDonagh *et al*, 1997).

Cachexia in Heart Failure

A wasting process similar to that observed in malignancy and other chronic diseases also develops in heart failure. Heart failure patients who develop cachexia have the greatest symptomatic limitation and poorest prognosis. In 1997 Anker *et al* found cachexia to be a strong independent predictor of reduced survival, with an 18 months mortality of 50% in heart failure patients with cachexia compared to 17% in those without cachexia (figure 1.1) (Anker *et al*, 1997). This relatively small, single centre study has become the cornerstone of cardiac cachexia research over the last 10 years.

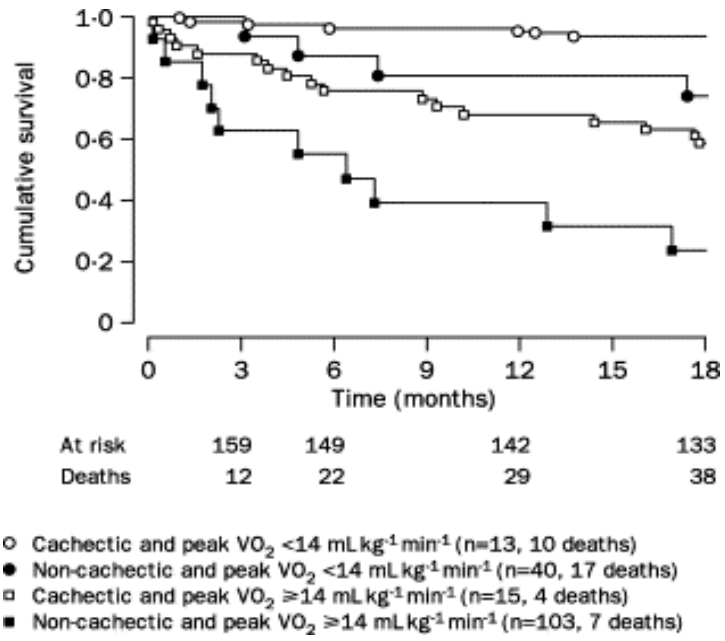


Figure 1.1. Kaplan-Meier survival and cumulative hazard curves for 18-month survival of 171 patients with CHF (Anker *et al*, 1997). Legend reproduced as originally published but incorrect. Should read: ○ Non-cachectic and peak VO_2 ≥14 mL.Kg^{-1}.min^{-1}; ● Cachectic and peak VO_2 ≥14 mL.Kg^{-1}.min^{-1}; □ Non-cachectic and peak VO_2 <14 mL.Kg^{-1}.min^{-1}; ■ Cachectic and peak VO_2 <14 mL.Kg^{-1}.min^{-1}.

The exact cause (or causes) of cardiac cachexia is unknown. Pro-inflammatory cytokines, increased neurohumoral activity, undernutrition, anabolic-catabolic hormone imbalance and immune activation have all been implicated (Levine *et al,* 1990; Anker *et al*, 1997; Anker *et al*, 1999).

Definition of cachexia

Early studies of cardiac cachexia specifically assessed percentage body fat and defined cachexia as <27% body fat in males and <29% in females (Murdoch *et al,* 1999). In chronic obstructive pulmonary disease (COPD) a definition of a body mass index (BMI) <23 kg/m^2 and a fat-free mass index (FFMI) <15 kg/m^2 in females and <16 kg/m^2 in males has been adopted, with the emphasis on loss of skeletal muscle (fat free mass) (American Thoracic Society and European Respiratory Society, 1999). The most commonly used operational definition of cachexia in recent heart failure studies derives from the study by Anker *et al*, as an unintentional weight loss of ≥7.5% body weight over a period of at least 6 months (Anker *et al,* 1997). While arbitrary, and providing no information on the relative change in body composition, this definition is easily applicable in the clinical environment and has been widely adopted. More recent post-hoc analysis of data regarding weight change during the Studies of Left Ventricular Dysfunction (SOLVD) trial suggested that >6% weight loss is a more powerful predictor of outcome (Anker *et al*, 2003).

Epidemiology of cardiac cachexia

There is limited knowledge of the incidence and prevalence of cachexia in heart failure. In the SOLVD (Studies of Left Ventricular Dysfunction) treatment study,

investigating the effect of enalapril in heart failure with left ventricular systolic dysfunction, 28% of patients had ≥7.5% weight loss during a mean follow-up of 35 months. Between 24 and 36 months follow-up the proportion of patients with ≥7.5% weight loss increased from 10% to 14% in the treatment group and 13% to 15% in the placebo group (Anker *et al*, 2003). Castillo-Martinez *et al* reported a 6 months incidence of cachexia (defined as weight loss >6% in 6 months) of 19% in a small, single centre study (Castillo-Martinez *et al*, 2005).

Systemic inflammation in cardiac cachexia

Heart failure is associated with elevated circulating levels of inflammatory mediators. Increased levels of the inflammatory cytokine tumour necrosis factor alpha (TNF-α) correlate with disease severity and may contribute to disease progression (Levine *et al*, 1990; Torre-Amione *et al*, 1996; Torre-Amione *et al*, 2000). Contrary to this hypothesis, anti-cytokine therapy in the form of etanercept, a TNF-α antagonist, or infliximab, an antibody to TNF-α, had no effect on clinical status, death or hospitalisation in patients with heart failure at 6 months follow-up (Chung *et al*, 2003; Mann *et al*, 2004).

The presence of a more pronounced inflammatory response, and more specifically higher circulating levels of TNF-α, has been observed in patients with cardiac cachexia. This has lead to the suggestion that the inflammatory cytokines may play a role in the wasting process. (Levine *et al*, 1990; McMurray *et al*, 1991; Dutka *et al,* 1993; Anker *et al,* 1997).

Tissue inflammation in cardiac cachexia

It is speculated that the presence of marked systemic inflammation may be associated with muscle inflammation and tissue cytokine activation. The only previous study quantifying muscle cytokine activation found increased expression of TNF-α, IL-1β and IL-6 in a heterogeneous population of patients with heart failure (Gielen *et al* 2003). A large proportion of the patients studied by Gielen *et al* had dilated, rather than ischaemic, cardiomyopathy, with no reported details regarding weight or body composition.

TNF-α is also produced by adipose tissue (Kern *et al*, 1995). It has been proposed that TNF-α plays a key role in the depletion of adipose tissue mass in cachexia by promoting lipolysis and inhibiting lipogenesis (figure 1.2) (Warne *et al*, 2003). Argiles *et al* argued that elevated circulating TNF-α levels, as opposed to local adipose tissue-derived TNF-α, is responsible for the metabolic alteration in adipose tissue in cachexia (Argiles *et al*, 1997).

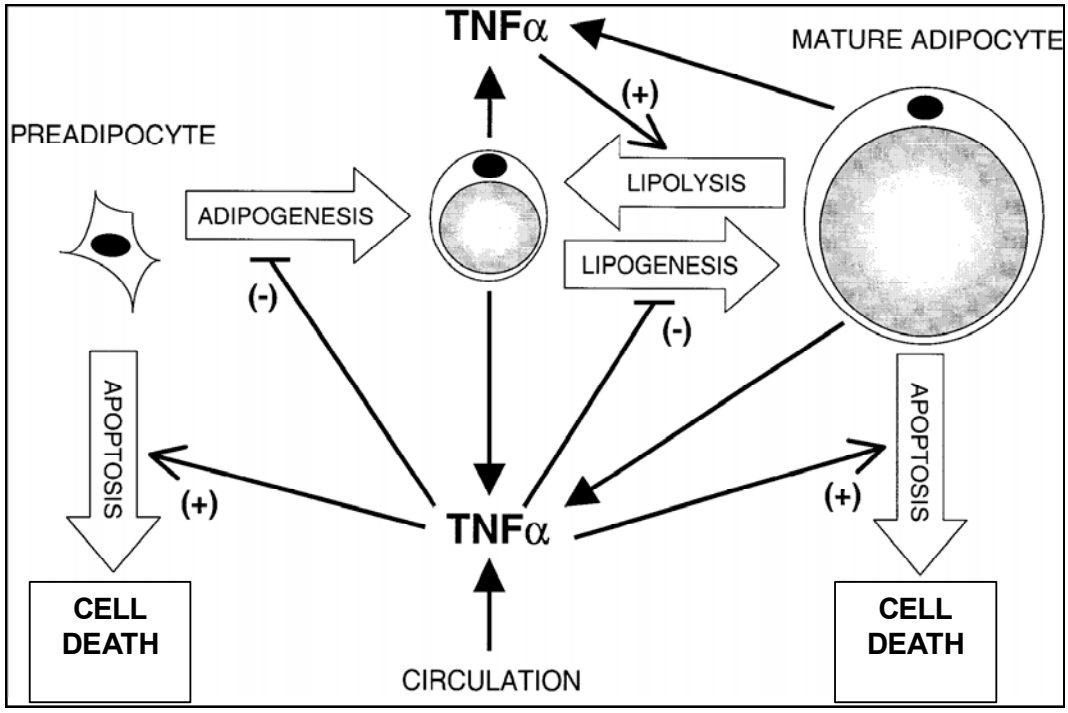


Figure 1.2. Proposed mechanisms by which TNF-α acts to decrease adipocyte mass. Reproduced and modified from Warne *et al*, 2003.

Energy metabolism in cardiac cachexia

Heart failure is associated with abnormal energy metabolism with decreased glucose oxidation and muscle glucose uptake, increased fat oxidation and levels of free fatty acids, and increased protein turnover (Norrelund *et al,* 2006).

Having previously been thought to act simply as an energy store, adipose tissue is now known to have endocrine and paracrine functions, producing hormones important in the regulation of energy metabolism. There has been particular interest in the more recently discovered leptin and adiponectin.

Leptin is a hormone predominantly produced by adipose tissue, which decreases food intake and increases energy expenditure. Obese individuals have increased leptin levels, and after weight loss leptin levels decline (Lonnqvist F *et al*, 1995; Maffei *et al*, 1995; Considine *et al,* 1996). In experimental studies leptin administration has been shown to regulate the production of inflammatory cytokines, and in humans TNF-α infusion increases serum leptin levels (Loffreda *et al,* 1998; Zumbach *et al,* 1997). It has therefore been suggested that leptin may play a role in the pathophysiology of cachexia.

While leptin levels have been found to be low in cachectic heart failure, patients with non-cachectic advanced heart failure show significantly higher serum concentrations of leptin than patients with moderate exercise intolerance, suggesting possible involvement of leptin in the progressive catabolic state in heart failure (Murdoch *et al,* 1999; Filippatos *et al,* 2000). Furthermore, in these patients with non-cachectic advanced heart failure, a strong positive relationship was found between serum levels of leptin and TNF-α (Schulze *et al,* 2003).

Adiponectin is a 244 amino-acid adipose tissue-specific peptide also thought to play an important role in the regulation of energy metabolism (Maeda *et al,* 1996; Chandran *et al,* 2003; Meier *et al,* 2004). In contrast to leptin, plasma adiponectin concentration is inversely correlated with body mass index and body fat in humans (Maeda *et al,* 1996; Chandran *et al,* 2003; Meier *et al,* 2004). Obesity is associated with reduced adiponectin levels, whereas levels are increased in anorexia nervosa (Iwahashi *et al,* 2003; Pannacciulli *et al,* 2003; Delporte *et al,* 2003). In experimental

animals, including genetically modified ones, administration of adiponectin reduces weight gain or leads to weight loss (depending on the model), possibly by increasing energy expenditure (Fruebis *et al,* 2001; Yamauchi *et al,* 2002; Masaki *et al,* 2003).

Adiponectin is thought to have anti-inflammatory and anti-atherogenic effects. It has been reported to negatively correlate with C-reactive protein (CRP) in male subjects with coronary artery disease (Ouchi *et al*, 2003). TNF-α has been suggested to both increase and decrease adiponectin secretion (Bruun *et al,* 2003; Degawa-Yamauchi *et al,* 2005; Carey *et al,* 2006; Wang *et al,* 2006). Reduced adiponectin concentration has been found in patients with acute coronary syndromes, although the association between adiponectin levels and stable coronary artery disease or subsequent cardiovascular risk appears to be modest (Nakamara *et* al, 2004; Sattar *et al*, 2006).

Adiponectin is also thought to regulate glucose and fatty acid metabolism, acting as an insulin sensitiser (Yamauchi *et al*, 2002; Wang *et al*, 2004). Levels of adiponectin are reduced in patients with type 2 diabetes mellitus (Hotta *et al*, 2000).

Plasma adiponectin concentration has recently been shown to be increased in heterogeneous populations of patients with heart failure and to be an independent predictor of outcome (Kistorp *et al,* 2005; George *et al,* 2006).

Natriuretic peptides in cardiac cachexia

The significance of natriuretic peptides in heart failure with left ventricular systolic dysfunction has been extensively studied. B-type natriuretic peptide (BNP) is well

established as a strong predictor of outcome and marker of disease severity (Yu *et* al, 1999; Troughton *et al*, 2000; Selvais *et al*, 2000; Berger *et al*, 2002, Lee *et al*, 2002). Although higher levels of BNP have been described in patients with more advanced heart failure, no relationship with cachexia has as yet been investigated.

Lipolysis is thought to be predominantly regulated by the stimulatory action of the sympathetic nervous system and catecholamines, and the inhibitory action of insulin. Catecholamines, through binding to β-receptors on the surface of adipocytes, and via a cyclic adenosine monophosphate (cAMP) dependent pathway, result in the phosphorylation, and hence activation, of lipase. Recent studies have demonstrated that beta-blockers, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) prevent lipolysis and weight loss (Lainscak *et al*, 2006; Anker *et al*, 2003; Cabassi *et al*, 2006).

Recently, a novel lipolytic and potential lipid-mobilising effect of natriuretic peptides has been identified (Sengenes *et al,* 2000). These actions appear to be mediated by specific adipocyte membrane receptors, which via a cyclic guanine monophosphate (cGMP) dependent pathway may indirectly stimulate adiponectin production. A role for BNP in the regulation of fat metabolism was supported by the recent finding by Horwich et *al* of an inverse relationship between BNP and BMI in patients with advanced heart failure (Horwich *et* al, 2006). Of further interest was the observation of Kistorp *et al* of a positive relationship between NT-proBNP and plasma adiponectin concentration in heart failure patients (Kistorp *et al*, 2005).

Skeletal Muscle Development, Injury and Repair

Skeletal muscle consists of muscle fibres, which develop when myoblasts proliferate, fuse and differentiate into myocytes. Skeletal muscle terminally differentiates and is therefore unable to replicate. However, some myoblasts persist in a quiescent state under the basal lamina of the muscle fibre as satellite cells (Mauro *et al*, 1961). These satellite cells retain the ability to proliferate, providing a mechanism for muscle growth and repair (figure 1.3). Impairment of this proliferative potential will limit the ability of a muscle to repair following injury from mechanical, oxidative or inflammatory stress. The number of satellite cells, and this ability to replenish, declines with age (Bischoff *et al*, 1994).

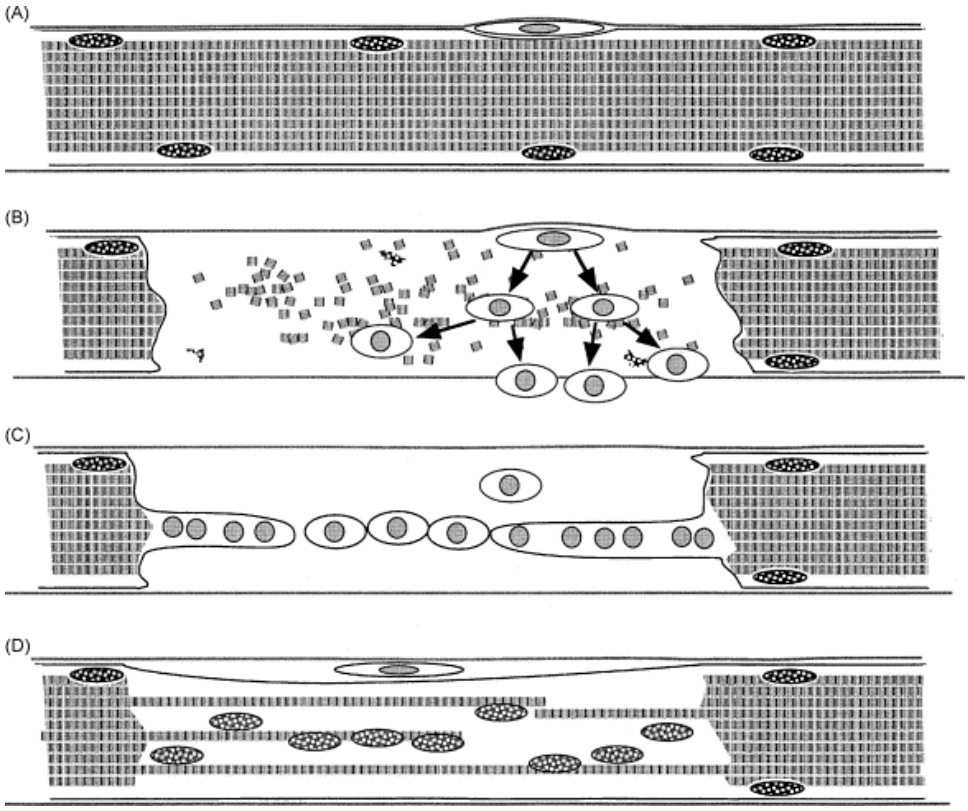


Figure 1.3. Regenerating muscle fibre. A) Queiscent satellite cell on basal lamina. B) Muscle fibre damage with satellite cell proliferation. C) and D) Myoblast fusion and differentiation into myocytes and myotubes. Diagram reproduced from unidentified source.

Satellite cells are activated by injury with a number of growth factors and cytokines involved in their proliferation, differentiation and chemotaxis (Chen *et al*, 1992; Tatsumi *et al,* 1998; Bischoff *et al,* 1997). More recent work in mice has also suggested that the environment of growth factors and cytokines at an injury site may have a detrimental effect by triggering the differentiation of cells into various cell lineages, including myofibroblasts, leading to fibrosis and poor functional recovery (Li *et al*, 2002).

Muscle development and regeneration is regulated at a molecular level by muscle regulatory factors (MRFs). This superfamily of transcription factors activate muscle specific genes. While quiescent satellite cells in rodent muscle do not express MRFs, when injured a fraction of satellite cells are activated, re-enter the cell cycle and express MRFs, before going on to differentiate, fuse and form muscle fibres (Grounds *et al*, 1992; Bischoff *et al,* 1986; Bischoff *et al,* 1994).

The cell cycle is regulated by activation and inactivation of cyclin-dependent kinases (CDKs). Inhibitors of CDKs can prevent cells entering the cell cycle (by holding them in the gap phase) and thereby reduce their proliferative capacity. The *Cip*/*Kip* family of CDK inhibitors (p21, p27 and p57) are the most important and ubiquitous. When cultured myoblasts are induced to differentiate this results in the upregulation of inhibitors of the cell cycle, including the cyclin dependent kinase (CDK) inhibitors (Walsh *et al*, 1997). Activation of the CDK inhibitor genes is thought to be regulated by the muscle regulatory factor MyoD (Guo *et al*, 1995).

MyoD is present in low levels in adult skeletal muscle. Following injury there is increased expression by satellite cells. MyoD deficient muscle has impaired ability to regenerate after injury. The transcription factor NF-κB has a key role in the activation of genes involved in inflammatory and proliferative responses, and is activated itself by TNF-α. Work in mice has suggested that NF-κB, activated by TNF-α, acts as a mediator of cytokine induced muscle degeneration by suppressing the expression of MyoD (Guttridge *et al*, 2000).

The molecular regulation of skeletal muscle wasting has been most extensively studied in sepsis, injury and cancer, with the mechanisms involved suspected to be similar in other catabolic conditions such as COPD and heart failure. A cascade of components and regulatory mechanisms appear to be involved in this complex pathway with gene expression of several components of the pathway known to be upregulated (Hasselgren *et al*, 2002).

Changes in Skeletal Muscle in Heart Failure

The major symptomatic limitation experienced by heart failure patients is exercise intolerance caused by breathlessness and fatigue. The extent of exercise intolerance has been shown to be unrelated to the degree of central haemodynamic disturbance and is thought partly to be attributable to changes in skeletal muscle and reduction in skeletal muscle mass.

Histological, metabolic, vascular perfusion, neurohumoral, inflammatory, and gene regulatory changes have been described in skeletal muscle of heart failure patients. There has been debate regarding whether these changes are related to heart failure *per se* or are due to disuse and deconditioning. Changes in skeletal muscle phenotype and the characteristics defining it's oxidative capacity (fibre type, oxidative enzymes, mitochondria, contractile proteins, capillary density) have been shown to relate to peak oxygen consumption and the degree of exercise intolerance (Wilson *et al,* 1993*;* Schaufeberger *et al*, 1995; Massie *et al*, 1996; Vescovo *et al,* 1996; Toth *et al*, 1997; Duscha *et al*, 2002; Drexler *et al*, 1992; Nicoletti *et al,* 2003).

Cachexia in Chronic Obstructive Pulmonary Disease

It has been suggested that common pathogenic mechanisms underlie different cachectic states. As in heart failure, cachexia in COPD is associated with increased susceptibility to exacerbations of clinical symptoms and severely impaired functional capacity. As discussed earlier, cachexia has been defined in COPD as a body mass index (BMI) <23 kg/m^2 and a fat-free mass index (FFMI) <15 kg/m^2 in females and <16 kg/m^2 in males, with the emphasis on loss of fat free mass (muscle mass) (American Thoracic Society and European Respiratory Society, 1999). A recent small, single centre study applying a hybrid definition of cachexia, as an unintentional weight loss of ≥7.5% body weight over at least 6 months and a BMI ≤24 kg/m^2, found the prevalence of cachexia in COPD to be 33% (Koehler *et al*, 2006).

As in heart failure, circulating TNF-α levels are elevated in patients with COPD, and even more markedly in the presence of cachexia (Di Francia *et al*, 1994; Takabatake *et al*, 2000). The presence of chronic hypoxia is thought to result in increased TNF-α production (Takabatake *et al*, 2000).

COPD is associated with recurrent infective and non-infective exacerbations. It is thought that the associated acute systemic inflammatory response results in recurrent inflammatory insults and injury to the skeletal muscle. Similarly, heart failure is complicated by recurrent acute decompensations, and although the mechanism principally involves haemodynamic and neurohumoral changes, there is an associated rise in markers of systemic inflammation, suggesting that a similar mechanism of

recurrent acute inflammatory insults could contribute to muscle wasting in heart failure (Mueller *et al,* 2006).

Our group, in investigating the molecular and cellular basis for skeletal muscle wasting in COPD, previously demonstrated that gene and protein expression of the cyclin dependent kinase (CDK) inhibitor p21 was increased in the skeletal muscle of COPD patients with cachexia (figure 1.4) (Fulds, 2004). We hypothesised that recurrent inflammatory insults in COPD resulted in muscle injury, and that local activation of cell cycle inhibitors impaired the muscles ability to repair. We further hypothesised that inflammatory cytokines act as a mediator of this process by influencing local gene expression. There are no previous studies of cell cycle inhibitor expression in the skeletal muscle of patients with heart failure.

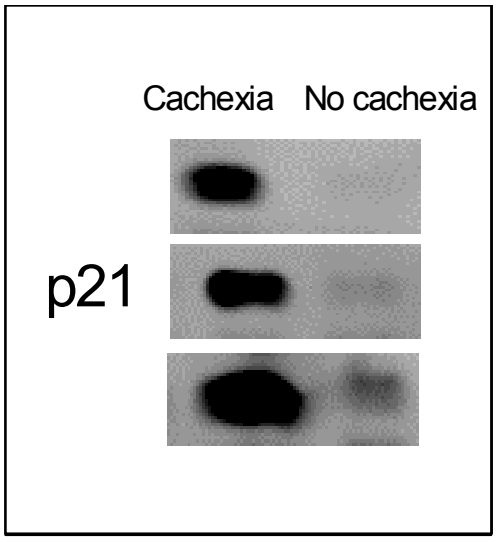


Figure 1.4. **Western blots of relative expression of p21 protein in skeletal muscle of cachectic and non-cachectic COPD patients** (Reproduced from Fulds, 2004).

Aim

The aim of this doctoral work was to characterise the nature of cachexia in patients with heart failure and stable coronary artery disease, to quantify the loss of muscle mass, and test the hypothesis that muscle wasting is mediated by the activation of tissue cytokines and cell cycle inhibitors.

CHAPTER 2

GENERAL METHODS

Subjects

Subject recruitment

Patients were recruited between December 2002 and July 2004 from the cardiology clinics of the North Glasgow University Hospitals and the primary care based heart failure nurse liaison service. Patient records were screened to identify subjects with heart failure (HF) and coronary artery disease (CAD) (with or without a history of weight loss), and a control group of patients with CAD but no heart failure. A large number of heart failure patients were screened to identify a population with cachexia. This was due to the relatively small proportion of heart failure patients with cachexia, a lack of awareness of cachexia as a prognostically important problem, and consequently the absence of regular body weight documentation in patient records, and the frailty of this patient population with the not inconsiderable demands of participating in the study. We specifically only studied patients with underlying coronary artery disease in order to control for differences in inflammatory activity related to differing aetiologies of heart failure.

The study population was recruited to look at levels of gene expression in skeletal muscle and therefore the sample size was based on a power calculation using information from a previous study by our group looking at gene expression in skeletal muscle of patients with chronic obstructive pulmonary disease (COPD). We calculated that 7 patients in each group would give us a power of 0.80 to show a significant difference at the 5% level with a one-sided t-test given a 3-fold difference in expression of mRNA or protein being measured.

Age and activity matched healthy controls were recruited from patient companions at the clinics and a university staff advertisement. As previous studies in this area have involved heterogeneous populations of heart failure patients, a further control group of patients with dilated, rather than ischaemic, cardiomyopathy was recruited as an amendment to the study protocol.

Following baseline assessment six patients with heart failure were found to have preserved left ventricular systolic function. One patient with heart failure and one patient with heart failure and cachexia withdrew consent.

Subject groups

We studied five groups of subjects. Three were groups of patients with stable coronary artery disease (CAD): 1) HF-cachexia - those with HF, reduced left ventricular systolic function and cachexia, n=10; 2) HF-no cachexia - those with HF, reduced systolic function but no cachexia, n=20; and 3) CAD - those with CAD, no symptoms of HF and preserved systolic function, n=10. The other subject groups were: 4) IDCM – patients with dilated cardiomyopathy, n=7; and 5) Controls – healthy controls, n=9.

Skeletal muscle analysis was performed in a subgroup of subjects: HF-cachexia, n=7; HF-no cachexia, n=14; CAD, n = 5; healthy controls, n = 5.

The IDCM group were recruited as an amendment to the study protocol and underwent limited characterisation and blood analysis, and no skeletal muscle analysis.

A further population of young healthy male subjects were specifically recruited for an additional study reported in chapter 6.

Subject Characterisation

Coronary artery disease (CAD): CAD was defined as a previously documented myocardial infarction or evidence of obstructive coronary artery disease on coronary angiography.

Heart failure (HF): HF was defined using the European Society of Cardiology (ESC) Task Force and American College of Cardiology/American Heart Association (ACC/AHA) Guidelines as described previously in more detail in chapter 1 (Swedberg *et al*, 2005; Hunt *et al*, 2005).

Cachexia: As discussed in chapter 1, cachexia has previously been defined in a number of ways. The arbitrary definition devised by Anker *et al*, of cachexia as an unintentional weight loss of ≥7.5% body weight over at least 6 months, has been the most commonly used operational definition in recent heart failure studies and therefore was the definition we elected to use in this study (Anker *et al*, 1997).

Healthy controls: Healthy control subjects had no significant medical history or regular prescribed medication, and were age and activity-matched to the patient groups.

Left ventricular function: Left ventricular ejection fraction (LVEF) was measured by radionucleotide ventriculography (RNVG). The LVEF was determined for all subjects using dual region of interest, except in the IDCM group in whom ventriculography had been performed prior to recruitment as part of an advanced heart failure assessment and LVEF determined using the single region of interest technique. RNVG is a reproducible method with inter-study variability of <5% and inter-observer variability reported to be as low as 2%. The technique used was equilibrium ventriculography which uses a sample of the patient's blood labelled with technetium (^{99m}Tc) to produce a blood pool acquisition of several hundred summated beats, gated to the QRS complex, and acquired in a 45° left anterior oblique projection. Healthy volunteers <55 years of age had echocardiographic assessment of LVEF using the regional wall motion score. Administration of a radioisotope in this subject group was considered inappropriate after discussion with the Administration of Radioactive Substances Advisory Committee (ARSAC).

Symptomatic status: Patients with HF had their degree of functional limitation quantified using the New York Heart Association (NYHA) classification. This system assigns patients to one of four functional classes, depending on the degree of effort needed to precipitate symptoms: patients may have symptoms of HF at rest (class IV), on less-than-ordinary exertion (class III), on ordinary exertion (class II), or only at

levels of exertion that would limit normal individuals (class I) (The Criteria Committee of the New York Heart Association, 1964).

Cardio-respiratory exercise testing: Symptom limited cardio-respiratory treadmill exercise testing was performed as an objective assessment of functional capacity. All the tests were supervised by the same doctor and technician. Subjects were instructed to exercise until they felt unable to continue. Exhaled air was analysed to determine metabolic gas exchange with a metabolic cart. Oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were determined on-line with breath-by-breath analysis. The peak VO_2 was averaged over the last 30 seconds of exercise. The respiratory exchange ratio (RER) was determined as the ratio of VCO_2/VO_2 Rates of fat and carbohydrate oxidation were calculated using the stoichiometric equations of Frayn (Frayn, 1983). Continuous 12-lead ECG monitoring and heart rate and blood pressure response were recorded.

The STEEP (Standardised Exponential Exercise Protocol) protocol was used (table 2.1) (Northridge et al, 1990). This was devised as a single protocol suitable for subjects with a wide range of exercise capacities, which could be applied to a bicycle, or as in this study, the treadmill. As illustrated in the table the STEEP protocol employs one minute, stepwise increments in speed or gradient but never both simultaneously. The benefit of this protocol, particularly in patients with significant limitation in exercise capacity, is that smaller increments make it less likely that the subject will stop early following a transition of workload. In protocols with larger increments in workload subjects are more likely to stop within 5-10 seconds of a

change in stage, resulting in the grouping of the subjects duration of exercise rather than a spread of exercise capacity. We anticipated that this protocol would enable patients with HF to continue to a faster and steeper stage, possibly attaining a higher peak O_2 consumption, producing a peak VO_2 with improved reliability and prognostic value. Despite the advantages of this type of protocol it is important to note that patients with HF have slowed oxygen kinetics and therefore require time for O_2 consumption to reach near steady state (time provided by protocols with longer stages and larger increments but not by shorter stages and smaller increments) and therefore a steady state would never be achieved (Brunner-La Rocca, 1999)

Time (min)	Speed (km h^{-1})	Slope (%)
1	2.4	0
2	3.2	0
3	3.2	1.5
4	3.2	3.0
5	4.0	3.0
6	4.0	5.0
7	4.0	7.0
8	4.8	7.0
9	4.8	9.0
10	4.8	11.0
11	5.6	11.0
12	5.6	13.0
13	5.6	16.0
14	6.7	16.0
15	8.0	16.0

Table 2.1. STEEP (Standardised Exponential Exercise Protocol) treadmill protocol (Northridge et al, 1990).

Body composition analysis: A weight history over the preceding 6 months or more was documented. Where there was uncertainty this was validated from hospital and heart failure nurse records. Body mass index (BMI) was calculated from body mass and height ($Kg.m^{-2}$). Waist circumference was measured using a flexible steel tape with the anatomical landmarks of midway between the lower rib cage and iliac crest, and midway between the xiphoid process and the umbilicus.

Body composition analysis can be performed using a number of techniques. The gold standard densitometry models using underwater weighing were felt inappropriate in the patient groups due to the cumbersome nature of this technique. Facilities for the dual-energy X-ray absorbitometry (DEXA) technique were not available. Percentage body fat and fat-free mass index (FFMI) were therefore determined using bioelectrical impedance analysis (BIA). Bioelectrical impedance was measured using a body composition analyser (TBF-300GS, Tanita Corp) with an operating frequency of 50 kHz at 800 μA. All resistance measurements were adjusted for stature. The BIA system has been validated in healthy subjects against underwater weighing, DEXA, and tritium dilution volume methods, and more recently in stable heart failure patients against DEXA and deuterium dilution (Uszko-Lencer *et al*, 2006).

Blood Analysis

Blood sampling and storage

All subjects had a fasting blood sample taken between 9 and 10 am. Some blood was aliquoted to plain and ethylenediaminetetraacetic acid (EDTA) blood collection tubes and sent for same-day analysis by the Western Infirmary clinical biochemistry and haematology laboratories for urea, electrolytes, creatinine, albumin, haemoglobin, white cell count and platelets. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) equation (O'Meara et al, 2006). The rest of the blood was placed on ice, allowed to clot for 20 minutes, and then centrifuged at 3000rpm for 15 minutes at 4°C. The serum or plasma was aliquoted to avoid subsequent freeze-thaw cycles and stored at -80°C until analysis. Blood for the measurement of plasma B-type natriuretic peptide (BNP) was collected in chilled tubes containing EDTA.

Measurement of serum cytokines and adipokines

Commercially available enzyme linked immunosorbent assay (ELISA) kits were used to measure serum concentrations of tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), adiponectin and leptin (Quantikine, R&D Systems).

A monoclonal antibody specific for the protein being quantified is pre-coated onto a 96-well microplate. Standards for a dilution curve were prepared. The standards and samples were pipetted into the wells and any of the protein present bound to the pre-coated antibody. After washing away any unbound substances in the wells an enzyme-linked monoclonal antibody specific for the protein was added to the wells.

Following a further wash to remove any unbound antibody-enzyme reagent, a substrate solution was added with colour development in proportion to the amount of protein bound to the wells. The colour development reaction was stopped and the intensity of the colour measured. All samples, standards and controls were assayed in triplicate. The optical density of each well was determined within 30 minutes of stopping the reaction using an automatic microplate reader set to 450 nm. The mean for the triplicate readings for each standard, control, and sample was calculated. The optical density for the standards was plotted against their known concentrations and the best curve drawn. The data was linearised and the protein concentration determined for each sample by plotting the absorbance on the standard curve.

Measurement of lipids and C-reactive protein

Cholesterol, triglycerides, very low-density lipoprotein cholesterol (VLDL cholesterol), low-density lipoprotein cholesterol (LDL cholesterol), high-density lipoprotein cholesterol (HDL cholesterol) and C-reactive protein (CRP) concentrations were measured by enzymatic colorimetric methods using a Hitachi 917 automated analyser (Roche kits).

Fasting glucose, insulin and insulin resistance

Fasting glucose and insulin were measured and the homeostasis model assessment (HOMA1-IR) index used to estimate insulin resistance (insulin resistance = (fasting glucose x fasting insulin)/22.5) (Matthews *et al*, 1985).

Measurement of plasma B-type natriuretic peptide (BNP)

Plasma BNP was measured using the Shionoria immunoradiometric kit (Schering CIS, West Sussex, England).

Skeletal Muscle Analysis

Percutaneous skeletal muscle biopsies

Percutaneous needle biopsies were obtained from the *vastus lateralis* muscle from a subgroup of subjects using the technique described by Bergstrom (Bergstrom, 1975). With the subject supine, the lateral aspect of the thigh was infiltrated subcutaneously with local anaesthetic over the mid point of *vastus lateralis*. Using aseptic technique a 5mm skin incision was made to allow passage of the Bergstrom needle through the subcutaneous fat and muscle capsule. Once in the muscle, tissue was aspirated through a side window into the hollow needle and excised. Haemostasis was established with light compression. The wound was covered with a sterile dressing. Feeling this was not an insignificant procedure for the study subjects to undergo, prior to commencement of the study, I myself had a muscle biopsy taken as a healthy control for another project in the department. All muscle biopsies were performed by myself. The biopsies were immediately snap frozen in liquid nitrogen and stored at −70°C.

Skeletal muscle histology

A small sample of muscle was prepared on cork, frozen in isopentane-cooled liquid nitrogen and stored at –70°C. Transverse sections were cut with a cryotome at –20°C and stained for alkaline stable adenosine triphosphatase (ATP-ase) for identification of fibre types I, IIA and IIB. A specialist skeletal muscle histopathologist performed the examination of the biopsies.

Ribonucleic acid (RNA) extraction

To process the stored muscle biopsies for RNA extraction the frozen samples were transferred to a liquid nitrogen-cooled mortar and pestle and ground to a fine powder under liquid nitrogen. The suspension (tissue powder and liquid nitrogen) was transferred into a liquid-nitrogen–cooled 2 ml micro-centrifuge tube and the liquid nitrogen allowed to evaporate but without allowing the samples to thaw.

For the extraction of RNA for reverse transcription and subsequent gene amplification with real-time polymerase chain reaction (RT-PCR) it was important to use a non-trizol based system. A fibrous tissue specific mini column kit (RNeasy Fibrous Tissue Mini Kit, Qiagen) was used. The recommended amount of starting tissue for RNA extraction from skeletal muscle using this technique is 30 mg. A 3 mm cube of muscle powder, which for most tissues weighs 25–35 mg, was therefore used. The manufacturers estimated yield is 5-10 µg RNA from 10 mg muscle tissue. The maximum binding capacity of the mini spin columns is 100 µg RNA and it was important that this was not exceeded or the yield of RNA would have been reduced.

Disruption of the plasma membranes of cells and organelles with the mortar and pestle was required to release the RNA contained in the muscle sample. Lysis buffer (300 µl Buffer RLT) was added to the disrupted sample, which was then homogenized using a syringe and 20-gauge needle. Homogenization shears high-molecular-weight genomic deoxyribonucleic acid (DNA) and other high molecular-weight cellular components.

590 µl RNase-free water and 10 µl proteinase K solution were added to the homogenate and mixed thoroughly by pipetting. The sample was then incubated in a water bath at 55 °C for 10 min to allow proteinase K to remove proteins.

The sample was centrifuged for 3 min at 10,000 g at 20–25 °C with the debris forming a pellet. The supernatant (approximately 900 µl) was pipetted into a new 1.5 ml micro-centrifuge tube. 0.5 volumes of ethanol (100%, approximately 450 µl) was added to the sample and mixed well by pipetting. The RNeasy Mini Spin Column, which contains a silica gel membrane for RNA binding, was placed in a 2ml collection tube. 700 µl of the sample was pipetted into the column and centrifuged through at 8000 g (10,000 rpm) for 15 sec and the flow-through discarded. This step was repeated with the remainder of the sample. The column and membrane was then washed with Buffer RW1 (350 µl) by centrifugation for 15 sec at 8000 g and again the flow-through discarded.

Traces of DNA may co-purify to the membrane and therefore, on-column DNase digestion was performed by mixing RNase-free DNase I (10 µl) with Buffer RDD (70 µl) and pipetting the mixture directly onto the membrane. The column was incubated on the bench top at room temperature for 15 min. Following the incubation, the column was again washed with Buffer RW1 (350 µl) to remove the DNase and contaminants by centrifuging for 15 sec at 8000 g and discarding the flow-through.

The column was then transferred into a new 2 ml collection tube. Two further washes were performed using Buffer RPE (500 µl), the first with centrifugation for 15 sec at

8000 g and the second for 2 min at 8000 g. The column was then transferred to another 2 ml collection tube and centrifuged at 10,000 g for 1 min to ensure the membrane was completely dried. This was important to ensure that no ethanol was carried over to the RNA elution.

To elute the RNA from the membrane the column was transferred to a new 1.5 ml collection tube. 30 µl RNase-free water was pipetted directly onto the membrane and the tube centrifuged for 1 min at 8000 g. This step was repeated adding a further 30 µl RNase-free water and centrifuging for a further 1 min.

Purified RNA was stored at –80 °C. The concentration of the RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. For this process the RNA samples were diluted (10 µl of RNA sample plus 490 µl of RNase-free water (1/50 dilution) and the absorbance measured in a 1 ml cuvette. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml.

Complementary DNA (cDNA) synthesis

Samples for subsequent RT-PCR were all reverse transcribed using the iScript cDNA synthesis kit (BioRad). The iScript reverse transcriptase is RNase H^+. It is premixed with an RNase inhibitor, to prevent degradation of the RNA template, and a blend of oligo(dT) and random primers. The reaction composition and protocol used are outlined in table 2.2.

Reaction Set Up	**Volume**
5x iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	Xµl
RNA template	Xµl
Total volume	20µl
Reaction Protocol	
5 minutes at 25˚C 30 minutes at 42˚C 5 minutes at 85˚C Hold at 4˚C	

Table 2.2. Complementary DNA synthesis.

Relative quantification of messenger RNA (mRNA) using real-time polymerase chain reaction (RT-PCR)

RT-PCR, by measuring product during the exponential phase of the reaction, can detect two-fold changes, while traditional PCR, by measuring product during the plateau phase once the reaction has stopped and product is degrading, can only detect ten-fold changes (figure 2.1).

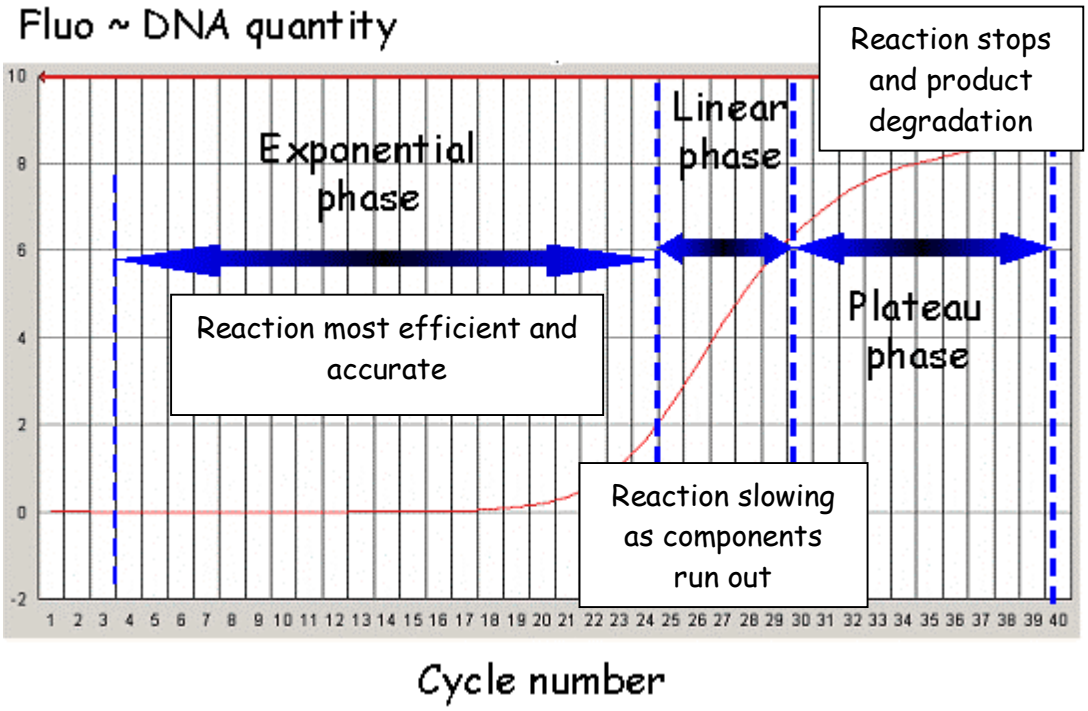


Figure 2.1. PCR amplification curve (Modified image from www.inra.fr).

We performed relative quantification of mRNA for our genes of interest using the iCycler iQ RT-PCR detection system (Bio-Rad). This is a 96-well plate set up with fluorescent detection during the exponential phase of target gene amplification. The threshold cycle is the point at which the reaction reached a fluorescent intensity above background.

Primer design

Primers were designed using the NCBI gene database and Primer 3 software. The following parameters were used:

Amplify a template 75-150 bp

Aim for a GC content of 50-60 %

Avoid G or C repeats longer than 3 bases

Place G's and C's on the ends of primers

Aim for a melting temperature (T_m) of 50-65 ˚C

Avoid complementarity between the forward and reverse primers (to avoid primer-dimer formation)

Avoid secondary structures

Verify specificity using Basic Local Alignment Search Tool (BLAST)

For some of the target genes we also used previously published primers to verify results. We analysed skeletal muscle expression of message for inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-18) and the cyclin dependent kinase (CDK) inhibitors (p21, p27 and p57). The primers used are listed in table 2.3.

Primer	Sequence 5' to 3'
B-actin Forward	GAC AGG ATG CAG AAG GAG ATT ACT
B-actin Reverse	TGA TCC ACA TCT GCT GGA AGG T
18S rRNA Forward	TAG AGG GAC AAG TGG CGT TC
18S rRNA Reverse	TGT ACA AAG GGC AGG GAC TT
B2M Forward	ATG AGT ATG CCT GCC GTG TGA
B2M Reverse	GGC ATC TTC AAA CCT CCA TG
CYC Forward	CCC ACC GTG TTC TTC GAC AT
CYC Reverse	CCA GTG CTC AGA GCA CGA AA
GAPDH Forward	CCA CCC ATG GCA AAT TCC
GAPDH Reverse	TGG GAT TTC CAT TGA TGA CAA
TNFα Forward	TGT GTT GTC CTT CCT GCA AC
TNFα Reverse	CTT GTA GGT GCC CAG GAG AG
IL-1β Forward	GGA CAA GCT GAG GAA GAT GC
IL-1β Reverse	TCG TTA TCC CAT GTG TCG AA
IL-6 Forward	AGC CCT GAG AAA GGA GAC ATG TA
IL-6 Reverse	CAT CTT TGG AAG GTT CAG GTT GT
IL-18 Forward	GGA ATT GTC TCC CAG TGC AT
IL-18 Reverse	GAA GCG ATC TGG AAG GTC TG
P21 Forward	ATG AAA TTC ACC CCC TTT C
P21 Reverse	CCC TAG GCT GTG CTC ACT T
P27 Forward	GGC TAA CTC TGA GGA CAC GC
P27 Reverse	TTG CAG GTC GCT TCC TTA TT
P57 Forward	TCG CTG TCC TCT CCT CTC TC
P57 Reverse	GCG CAC TAG TAC TGG GAA GG

Table 2.3. Primer sequences.

Reaction components and protocols

We used the iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCI, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50 units/ml, 6 mM MgCl2, SYBR Green I, 20 nM fluorescein, and stabilizers). SYBR green binds to double stranded DNA (dsDNA) product with detectable fluorescence increasing as the amount of product increases. The iTaq DNA polymerase is a hot-start enzyme activated by an initial 3 min denaturation step at 95 °C. This prevents non-specific amplification during plate preparation.

The reaction composition and a typical protocol are outlined in table 2.4. After the initial hot start step to activated the DNA polymerase a 2-step cycle of amplification with 50 repeats was used. Fluorescence was measured automatically at the end of step 2. Figure 2.1 shows an example of an amplification curve.

Reaction Set Up	**Volume**				
IQ SYBR Green Supermix	12.5µl				
Forward Primer	0.5µl				
Reverse Primer	0.5µl				
Sterile water	8.5µl				
CDNA template	3µl				
Total Volume	25µl				
Protocol	**Repeats**	**Step**		**Time**	**Temp**
Hot start	1	1		3 min	95˚C
Amplification	2	50	1	10 sec	95˚C
			2	45 sec	60˚C
Melt curve	3	1		1 min	95˚C
	4	1		1 min	55˚C
	5	80		10 sec	55˚C

Table 2.4. Real Time Polymerase Chain Reaction (RT-PCR) Protocol.

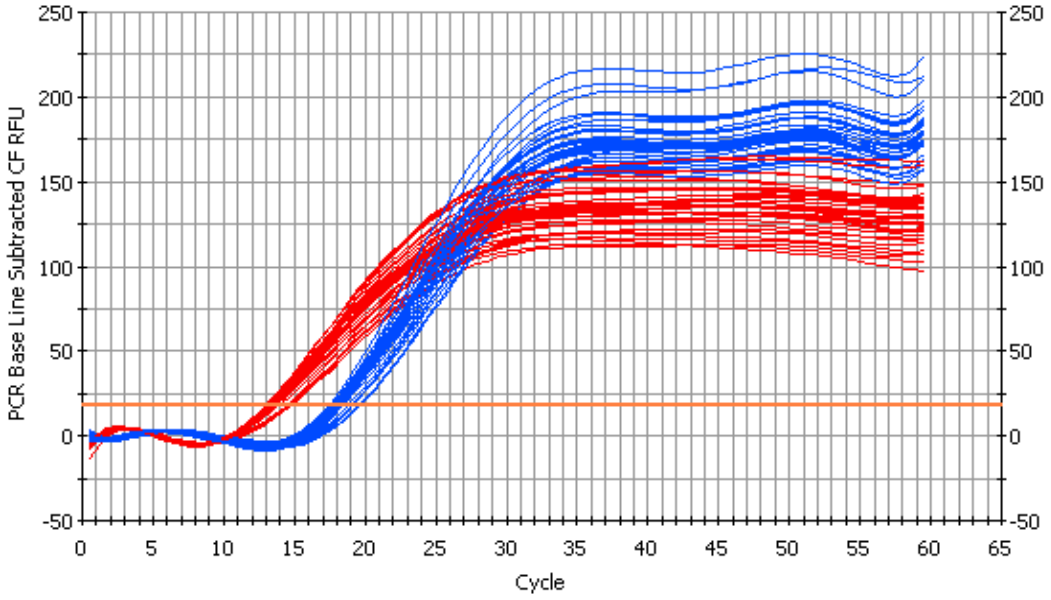


Figure 2.2. Example of target gene and housekeeping gene amplification curve.

After amplification was complete, the reaction was stopped, and the temperature of the samples reduced to 55 ˚C. The temperature was then increased by 0.5 ˚C every 10 seconds until the product of the reaction melted. All products from a particular primer should melt at the same temperature allowing the identification of any contaminating products such as primer-dimers. Figure 2.2 shows an example of a desirable melt curve.

To optimise the annealing temperature for primers a gradient protocol was performed. This involved a protocol similar to that illustrated in table 2.4 but with a 10 ˚C range of annealing temperatures applied down the rows of the plate (ie. Annealing at 50 ˚C in row A, 51.25 ˚C in row B, 52.5 ˚C in row C, increasing to 60 ˚C in row H) to identify which annealing temperature resulted in optimal amplification.

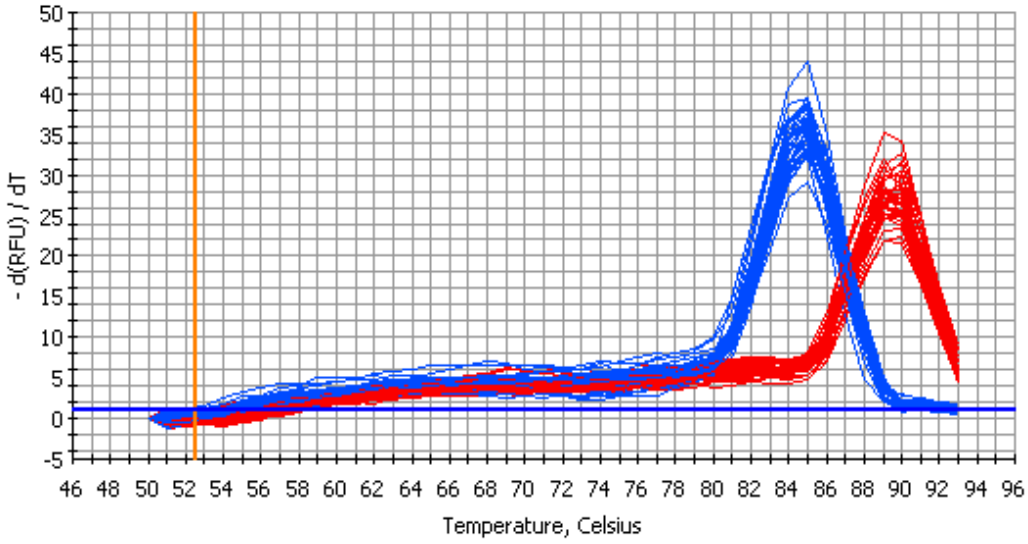


Figure 2.3. Target and housekeeping gene amplification product melt curves.

Target gene expression was measured relative to the expression of a house keeping gene. Each sample was run in triplicate for both the target and housekeeping gene amplifications. The threshold cycles of the triplicates were averaged and the quantity of the target gene expressed relative to the housekeeping gene for each sample in relative units.

Housekeeping gene

The selection of a housekeeping gene against which the relative expression of the target genes could be measured in skeletal muscle was problematic. The most commonly used housekeeping genes are muscle derived due to the usual stability of their expression. We selected beta-actin (β-actin), which has previously been widely used. We were concerned that the expression of β-actin would be altered (and most likely up-regulated) in the presence of inflammation in skeletal muscle and that this may mask any up-regulation in expression of the genes of interest.

In the absence of an 'ideal' house-keeping gene against which changes in target gene expression in skeletal muscle could be measured, we addressed this potential problem by selecting four further established house-keeping genes to allow comparisons of relative expression (CYC, B2M, GADPH and 18S ribosomal RNA). The 18S rRNA has previously been used to look at relative expression of inflammatory mediators in skeletal muscle (Gielen *et al*, 2003). The expression of TNFα was calculated relative to the expression of CYC, B2M, GADPH, and 18S ribosomal RNA in addition to β-actin. There was no between group difference in TNFα mRNA expression when calculated relative to each of the house-keeping genes.

Protein quantification using western blotting

Sample preparation

The disrupted muscle biopsy samples (tissue powder) were lysed and homogenised with a lysis buffer (120 µl) (table 2.5) plus protease inhibitor cocktail (1 tablet/10 mls lysis buffer) and using a syringe and 20-gauge needle.

Tris HCl (pH 7.4)	50mM	100mls of 100mM
EDTA	1mM	2mls of 100mM
DTT	50mM	
SDS	0.1%	2mls of 10%
Igepal	0.5%	1mls
Total volume		200mls

Table 2.5. Lysis buffer for preparation of muscle samples.

The protein concentration of the samples was determined using the Protein Assay ESL kit (Roche). A series of standard samples were prepared with a BSA protein standard (2 mg/ml) (table 2.6). The biopsy samples were diluted by adding 10 µl of sample to 190 µl lysis buffer (1:20 dilution). 50 µl of each of the standards and samples were added to a series of 1.5 ml cuvettes containing100 µl of Reagent A. After mixing they were incubated at room temperature for at least 5 minutes. Taking each cuvette in turn, 1000 µl of Reagent B was added, mixed, and absorbance measured at 485 nm against a lysis buffer zero reference, at *exactly* 30 seconds. A standard curve of absorbance versus concentration was plotted and from this the protein concentration of the samples was calculated (x 20 dilution). The same standard curve was used for all samples validating it on each occasion with the absorbance for standard 1.

Standard	Lysis buffer	BSA protein standard	Concentration
1	400µl	0µl	0µg/ml
2	390µl	10µl	50µg/ml
3	380µl	20µl	100µg/ml
4	340µl	60µl	300µg/ml
5	300µl	100µl	500µg/ml
6	250µl	150µl	750µg/ml

Table 2.6. Preparation of standard samples for protein quantification.

60 µg of protein was used for each sample. Each sample was diluted at least 1:2 with sample buffer (table 2.7) plus β-mercaptoethanol (475 µl sample buffer plus 25 µl β-ME) to a maximum loading volume of 25 µl. The diluted samples and a protein ladder (15 µl) were incubated at 95 C for 5 minutes.

dH_2O	3.55ml
0.5M Tris-HCl (pH 6.8)	1.25ml
Glycerol	2.5ml
10% SDS	2.0ml
0.5% bromophenol blue	0.2ml
Total volume	9.5ml

Table 2.7. Sample buffer preparation.

Protein separation

Protein separation was performed using polyacrylamide gel electrophoresis with the SDS-PAGE (Laemmli) Discontinuous Buffer System. This system denatures proteins by heating them in buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (βME). The resultant polypeptides separate according to their molecular weights.

Gel preparation and formulation: A glass plate sandwich with casting frame and stand (Mini-PROTEAN 3, BioRad) was used to cast the gels. 10% resolving gels and 4% stacking gels were used (table 2.8). The resolving gel buffer was 1.5 M Tris-HCl (pH 8.8) and stacking gel buffer 0.5 M Tris-HCl (pH 6.8).

After the 10% resolving gel was prepared as shown in table 2.9, 10% APS (50 µl) and TEMED (5 µl) were added to the gel immediately prior to pipetting. The mixture was swirled gently to initiate polymerisation. The resolving gel solution was pipetted to 1 cm below the level reached by the stacking gel comb teeth, and immediately overlaid with dH_2O. The gel was left for 45 minutes to polymerise, after which the dH_2O was removed.

The 4% stacking gel was then prepared as shown in table 2.8 and, as for the resolving gel, 10% APS (50 µl) and TEMED (10 µl) were added just prior to pipetting. The stacking gel solution was pipetted directly on top of the resolving gel until the top of

the short plate was reached. The comb was then inserted and the gel left for 45 minutes to polymerise, after which the comb was removed.

% gel	DH_2O	30% Acrylamide/Bis	Gel Buffer	10% SDS
10%	4.1ml	3.3ml	2.5ml	0.1ml
4%	6.1ml	1.3ml	2.5ml	0.1ml

Table 2.8. Resolving and stacking gels for protein separation.

Tris 25mM	3.94g
Glycine 192mM (20% methanol)	14.4g
Dissolve in dH_2O	
Add 200ml of methanol	
Adjust volume to 1000ml with dH_2O	

Table 2.9. Towbin buffer for protein transfer.

Electrophoresis: The gel cassette sandwich was removed from the casting stand and frame and transferred to the electrophoresis module. It was placed on the electrode assembly and then together they were slid into the clamping frame. The assembled inner chamber was then placed into a mini-tank.

Electrophoresis running buffer was prepared by diluting stock solution (10 x Tris/Glycine/SDS, 50 ml in 450 mls dH_2O). The inner chamber was filled with the buffer until the level was half way between the tops of the short and long plates (approximately 125 ml) and approximately 200 ml of the buffer was added to the mini-tank.

The samples were loaded slowly into the wells using a loading guide and pipette with gel loading tips. The gel was then run at 200 V for 35 minutes

Protein transfer

After electrophoresis was complete the inner chamber was removed, running buffer discarded, and the gel cassette sandwich removed. The gel was then removed from the sandwich by gentle prising apart the glass plates and removing the larger plate using the gel releaser tool. After cleaving the stacking gel section the gel and plate were soaked in pre-chilled towbin transfer buffer (500 ml) for 15 minutes to allow equilibration (table 2.9).

A nitrocellulose transfer membrane and blotting paper (x 3) were cut to the dimensions of the gel (8.3 x 5 cm) always handling with forceps or gloves. The membrane was soaked briefly in methanol and then in towbin transfer buffer for 15-30 minutes, sliding the membrane in slowly to avoid traping air bubbles. After saturating briefly in towbin a blot was placed on the transfer plate (Trans-Blot SD semi-dry electrophoretic transfer cell). A pipet was rolled over the surface of the paper to remove any trapped air bubbles. The membrane was overlaid and any air bubbles rolled away. The gel was then floated off the small glass plate by agitating gently and placed onto the membrane, and any air bubbles rolled away. The other 2 blots were briefly soaked in the towbin and then placed on top, again rolling away any air bubbles between each layer. The gel was transferred at 15 V for 30 minutes. The membrane was then removed rinsed twice and soaked in phosphate buffered saline (PBS) (table 2.10).

KH_2PO_4	0.144g	1.06mM
$K_2HPO_47H_2O$	0.795g	2.96mM
NaCl	9.00g	155mM
Dissolved in 800ml dH_2O		
PH adjusted to 7.4		
Dilute to 1000ml with dH_2O and autoclave		

Table 2.10. Phosphate buffered saline (PBS) for protein detection.

Protein detection

The primary antibody for specific protein detection was reconstituted in 500 µl PBS to a concentration of 1 µg/µl. The transfer membrane was incubated in a blocking agent (100 ml PBS plus 3-5% powdered milk), for at least 30 minutes at room temperature (or overnight at 4 C) with constant agitation, to block non-specific protein binding sites. The antibody was diluted 1:1000 in the blocking agent (30 µl in 30 ml), and the membrane incubated in this solution for a further 1.5-2 hours at room temperature (or overnight at 4 C) with constant agitation. The membrane was then washed 5 times for 5 minutes with wash buffer (250 ml PBS plus 125 µl Tween) with constant agitation. The primary antibodies used were monoclonal anti-human TNF-α antibody, monoclonal anti-human IL-1β antibody, monoclonal anti-human IL-6 antibody, and monoclonal anti-human p21 antibody (R&D Systems).

The secondary antibody (Goat anti-Mouse IgG, Upstate), conjugated to the enzyme horseradish peroxidase (HRP), was reconstituted and diluted 1:1000 in the blocking agent. The membrane was then incubated in this solution for 1 hour at room

temperature (or overnight at 4 C) with constant agitation. The membrane was washed a further 5 times with wash buffer and then rinsed quickly 5 times with dH_2O.

Chemiluminescent HRP substrate luminol reagent (LumiGLO, Upstate) was prepared by combining 2.5 ml of substrate solutions A and B protecting the reagent from light and allowing to warm to room temperature. The membrane was transferred to a fresh tray with forceps and the LumiGLO added, incubating for 1 minute at room temperature. The membrane was removed, excess reagent drained by touching the corner to filter paper, and placed in a film exposure folder, ensuring there were no air bubbles between the membrane and folder. The folder was placed in an X-ray film exposure cassette. The X-ray film was then exposed to the membrane in a dark room (exposure time varying from a few seconds to a few minutes) and the film developed.

Statistical Techniques

GraphPad Instat (GraphPad Software Inc., San Diego, USA) software was used for all statistical calculations.

Mean and medians

Results are expressed as mean (SD) for subject characteristics and median (range) for biomarkers. Normality testing was performed and parametric or non-parametric tests were then used as appropriate. Data not normally distributed was log transformed to allow parametric testing where appropriate . Two-sided tests of significance were used. Statistical significance was taken at the 5% level.

Comparisons

Differences between groups were assessed using one-way analysis of variance (ANOVA) or Kruskal-Wallis non-parametric ANOVA. Bartlett's test for homogeneity of variance was performed. Where differences were identified a Dunnett's (parametric) or Dunn's (non-parametric) multiple comparison *post-hoc* test was performed. Fisher's exact test was used to compare categorical characteristics.

Correlations

The data was logarithmically transformed for linear regression analysis and Run's test used for departure from linearity. Relationships were further assessed by multiple linear regression analysis allowing adjustment for multiple relevant factors.

CHAPTER 3

THE DEVELOPMENT OF CACHEXIA IN HEART FAILURE PREDOMINANTLY INVOLVES THE LOSS OF FAT WITH THE RELATIVE PRESERVATION OF SKELETAL MUSCLE AND BONE

Introduction

Although cachexia has been identified as a prognostically important development in patients with heart failure, its definition and pathophysiology remain the subject of debate (Von Haehling *et* al, 2007).

As discussed in the preceding chapters, the most commonly used definition of cachexia in recent heart failure studies has been the involuntary loss of a percentage of body weight over a fixed period of time (Anker *et al,* 1997). In the absence of detailed information regarding body composition it has commonly been assumed that muscle wasting is the major contributor to this weight loss.

One of the most frequently postulated causes of cachexia is cytokine activation and, in particular, increased production of tumour necrosis factor alpha (TNFα). Ageing, muscular dystrophy, and other chronic diseases have been associated with activation of cell cycle inhibitors (cyclin dependent kinase (CDK) inhibitors), resulting in impaired proliferation of muscle progenitor cells and muscle repair (Welle *et al,* 2004; Endesfelder *et al,* 2000; Dasarathy *et al,* 2004; Fulds, 2004). We hypothesised that the presence of more markedly elevated levels of circulating cytokines in cardiac cachexia would be associated with increased muscle cytokine expression. We further hypothesised that activation of CDK inhibitors in skeletal muscle, possibly under the regulation of the inflammatory cytokines, may also be involved in the development of cardiac cachexia.

To further investigate the nature and pathogenesis of cardiac cachexia we assessed body composition and measured circulating levels of inflammatory cytokines, and skeletal muscle expression of cytokines and cell cycle inhibitors in a well characterised population of heart failure patients with and without cachexia.

In order to control for differences in cytokine activation and inflammatory activity related to differing aetiologies of heart failure, we specifically only studied patients with underlying coronary artery disease. We also studied a control group with stable coronary artery disease, with normal ventricular function and without heart failure, as well as a group with idiopathic dilated cardiomyopathy, and healthy controls.

Methods

Subject recruitment

As discussed in more detail in chapter 2, subjects were recruited between December 2002 and July 2004 from cardiology clinics at the North Glasgow University Hospitals and the primary care based heart failure nurse liaison service. Patient records were screened to identify subjects with heart failure (HF) and coronary artery disease (CAD), with or without a history of weight loss, a control group with CAD but no HF, and a group with idiopathic dilated cardiomyopathy (IDCM). Age and activity matched healthy controls were recruited from patient companions at the clinics and a university staff advertisement. Informed written consent was obtained from all participants and the study approved by the local ethics committee.

For recruitment, cachexia was defined as an unintentional weight loss of ≥7.5% body weight over ≥6 months (Anker *et* al, 1997). CAD was defined as a documented myocardial infarction or the presence of coronary artery disease on coronary angiography.

Subject groups

Five groups of subjects were studied. Three were groups of patients with stable coronary artery disease (CAD): 1) HF-cachexia - patients with CAD, heart failure (HF), reduced left ventricular (LV) systolic function and cachexia, n=10; 2) HF-no cachexia - those with CAD, HF, reduced LV systolic function but no cachexia, n=20; and 3) CAD - those with CAD, but no symptoms of HF and preserved LV systolic

function, n=10. The other subject groups were: 4) IDCM - patients with dilated cardiomyopathy, n=7; and 5) HC - healthy controls, n=9.

Heart failure characterisation and body morphology

Subjects were characterised by New York Heart Association (NYHA) classification, left ventricular ejection fraction (LVEF) and peak oxygen consumption (VO_2), obtained from symptom limited treadmill stress testing using the STEEP (Standardised Exponential Exercise Protocol) protocol (Northridge *et* al, 1990). The details of subject characterisation were previously discussed in detail in chapter 2. Co-morbidity and drug therapy were recorded.

A weight history, body mass index (BMI) and waist circumference were documented. Percentage body fat and fat free mass index (FFMI) were determined using bioelectrical impedance (BIA) with a body composition analyser (TBF-300GS, Tanita Corp).

Characterisation of the IDCM group was performed during advanced heart failure assessment as an amendment to the study protocol and was limited.

Blood sample collection and analysis

All subjects had fasting blood samples taken between 9 and 10am. Blood samples were placed on ice, allowed to clot for 20 minutes and then centrifuged at 3000 rpm for 15 minutes at 4°C. The serum or plasma was aliquoted and stored at -80°C until analysis. Serum tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were

measured in triplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikines, R&D systems). C-reactive protein (CRP) concentration was measured using a Hitachi 917 analyser (Roche). The methods used have been described in more detail in chapter 2.

Skeletal muscle analysis

Skeletal muscle analysis was performed in a subgroup of subjects: HF-cachexia, n=7; HF-no cachexia, n=14; CAD, n = 5; controls, n = 5. Percutaneous needle biopsies were obtained from the middle part of the *vastus lateralis* muscle using the Bergstrom technique (Bergstrom, 1975). The biopsies were snap frozen in liquid nitrogen immediately after they were excised and stored at −80°C until analysed.

A small sample of muscle was prepared on cork, frozen in isopentane-cooled liquid nitrogen and stored at –70°C. Transverse sections were cut with a cryotome at –20°C and stained for alkaline stable adenosine triphosphatase (ATP-ase) for identification of fibre type composition. Percentage area of type I, IIA, and IIB fibres, fibre thickness and muscle area (expressed as a percentage of the microscopic field) were analysed by a skeletal muscle pathologist.

Ribonucleic acid (RNA) was extracted from the skeletal muscle using a commercially available kit (RNeasy Fibrous Tissue Mini Kit, Qiagen). The RNA was reverse transcribed to produce complementary deoxyribonucleic acid (cDNA) (iScript cDNA synthesis kit, BioRad). Real-Time Polymerase Chain Reaction (RT-PCR) was used to relatively quantify the expression of cytokine messenger RNA (mRNA) in skeletal

muscle (BioRad iCycler iQ RT-PCR detection system with iQ SYBR Green Supermix). Primers were designed using the NCBI gene database and Primer 3 software (table 3.1). Cytokine and CDK inhibitor gene expression were measured relative to expression of the housekeeping gene β-actin. Samples were run in triplicate for both the target and housekeeping gene amplifications. The threshold cycles of the triplicates were averaged and the quantity of the target gene expressed as a ratio relative to the housekeeping gene for each sample.

Primer	**Sequence 5' to 3'**
B-actin Forward	GAC AGG ATG CAG AAG GAG ATT ACT
B-actin Reverse	TGA TCC ACA TCT GCT GGA AGG T
TNFα Forward	TGT GTT GTC CTT CCT GCA AC
TNFα Reverse	CTT GTA GGT GCC CAG GAG AG
IL-1β Forward	GGA CAA GCT GAG GAA GAT GC
IL-1β Reverse	TCG TTA TCC CAT GTG TCG AA
IL-6 Forward	AGC CCT GAG AAA GGA GAC ATG TA
IL-6 Reverse	CAT CTT TGG AAG GTT CAG GTT GT
IL-18 Forward	GGA ATT GTC TCC CAG TGC AT
IL-18 Reverse	GAA GCG ATC TGG AAG GTC TG
p21 Forward	ATG AAA TTC ACC CCC TTT C
p21 Reverse	CCC TAG GCT GTG CTC ACT T
p27 Forward	GGC TAA CTC TGA GGA CAC GC
p27 Reverse	TTG CAG GTC GCT TCC TTA TT
p57 Forward	TCG CTG TCC TCT CCT CTC TC
p57 Reverse	GCG CAC TAG TAC TGG GAA GG

Table 3.1. Primer sequences.

Cytokine and CDK inhibitor protein quantification was performed in a selection of skeletal muscle samples using the western blotting technique.

The methods used for skeletal muscle analysis have been described in more detail in chapter 2.

Statistical analysis

Subject characteristics and measures of body morphology are expressed as mean (SD). Biomarkers are expressed as median (range). Normality testing and Bartlett's test for homogeneity of variance were performed and differences between groups were assessed by one-way analysis of variance (ANOVA) or Kruskal-Wallis non-parametric ANOVA. Where differences were identified Dunnett's or Dunn's multiple comparison *post hoc* test was performed. Fisher's exact test was used to compare categorical characteristics. The data was logarithmically transformed for linear regression analysis and a Run's test used for departure from linearity. Relationships were further assessed by multiple linear regression analysis adjusting for age, peak VO_2, LVEF, and estimated glomerular filtration rate (eGFR). Statistical significance was taken at the 5% level.

Results

Subject characteristics

Patients with HF-cachexia were older than the other subjects, but the difference was only significant in comparison to the IDCM group (table 3.2). The HF-cachexia group had worse functional class and lower peak VO_2 than the HF-no cachexia group. The IDCM group had better functional class, but lower peak VO_2 than the HF-cachexia group, although neither difference was significant. LVEF and peak VO_2 were lower in patients with HF (HF-cachexia and HF-no cachexia groups) than CAD patients and healthy controls.

Patients with HF-cachexia had the shortest duration of exercise: HF-cachexia 124.6 (101) seconds, HF-no cachexia 580.3 (327.4) seconds, CAD 585.6 (178.6) seconds, and HC 674 (214.6) seconds ($p<0.01$ for HF-cachexia compared to each other group) (figure 3.1). In patients with HF (HF-cachexia and HF-no cachexia groups), duration of exercise was lower in those with NYHA class IV than in those with NYHA class III or II symptoms: NYHA class IV 40.7 (20.2) seconds, NYHA class III 280.9 (206.2) seconds, and NYHA class II 750 (282.1) seconds ($p<0.01$ for NYHA IV compared to NYHA III and II) (figure 3.2).

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	IDCM (n=7)	HC (n=9)
Mean age (years)	72.7	67.6	64.4	52.4**	59
Gender (M/F)	8/2	18/2	9/1	6/1	4/5
NYHA II/III/IV	0/7/3	12/8/0	-	5/2/0	-
LVEF (%)	27.1 (11.9)	35.4 (9.7)	73.0 (6.4)**	15.0 (4.0)	68.8(5.4)**
Peak VO_2 (mL/min/Kg)	9.8 (2.3)	13.6 (3.9)*	21.2 (2.0)††	9.2 (2.0)	26.8 (5.0)††

Table 3.2 Subject characteristics. LVEF (%) measured using dual region of interest (normal ≥55%), except IDCM group where single region of interest technique used (normal ≥40%). Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. Categorical characteristics compared with Fisher's exact test. For HF-cachexia vs each other group: *$p<0.05$, **$p<0.01$, ***$p<0.005$, †$p<0.001$, ††$p<0.001$.

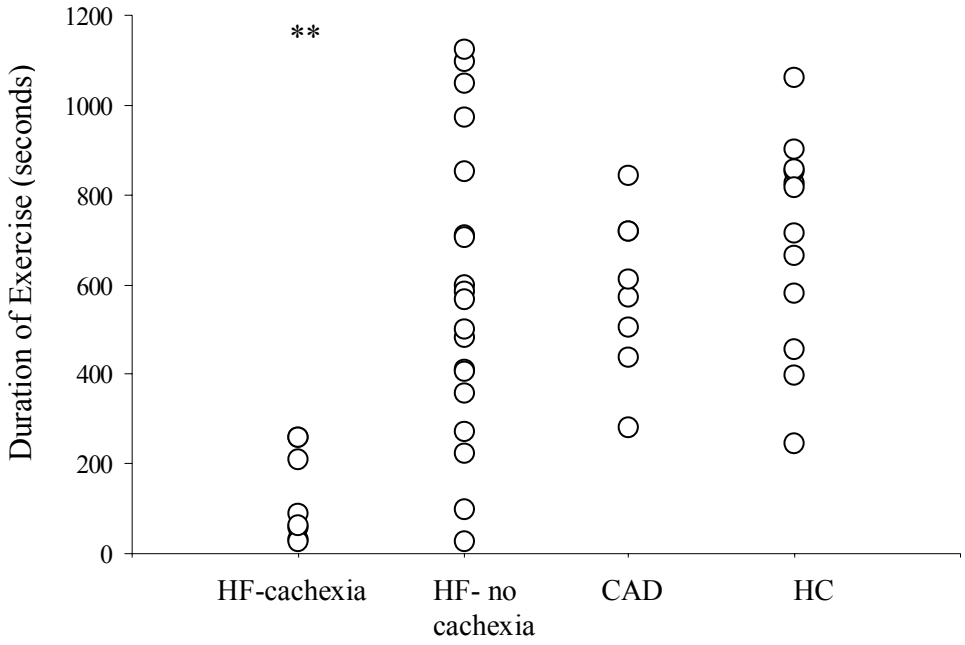


Figure 3.1. Duration of exercise by subject group. Differences between groups assessed by ANOVA. *Post hoc* analysis with Dunnett's multiple comparisons. For HF-cachexia vs each other group: **p<0.01.

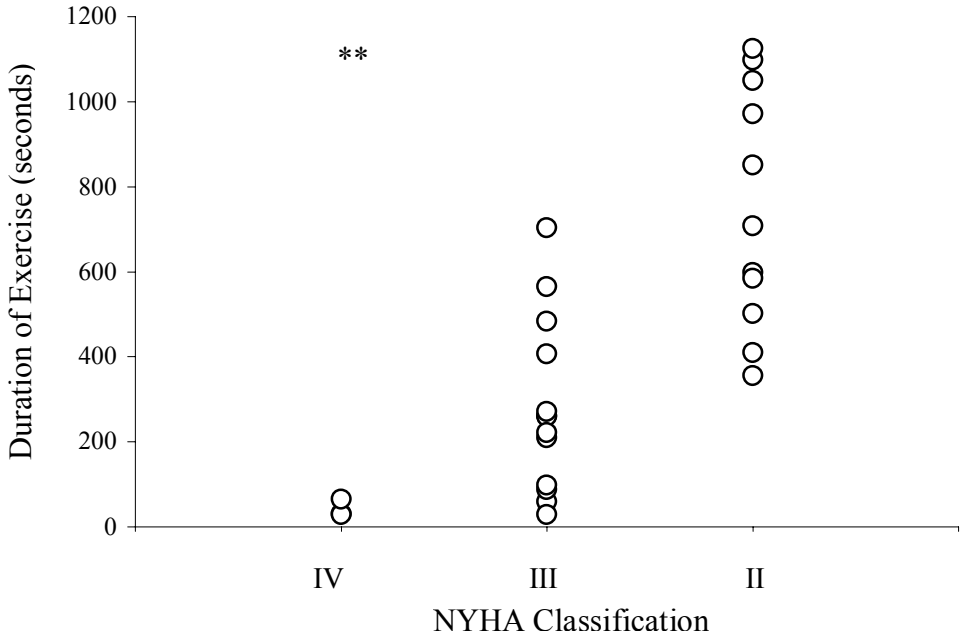


Figure 3.2. Duration of exercise by NYHA Classification. Categorical characteristics compared with Fisher's exact test. For NYHA IV vs III and II: **p<0.01.

As discussed in chapter 2 we decided to use the STEEP (Standardised Exponential Exercise Protocol) protocol, which by employing one-minute stepwise increments in speed or gradient, is thought to be more appropriate in patients with significant limitation in exercise capacity. The smaller increments are proposed to make it less likely that subjects will stop early following a transition of workload, avoiding grouping of subjects' duration of exercise and elucidating a spread of exercise capacity. While we continued to observe some grouping around the 60 seconds increments there was a reasonable spread of exercise duration as illustrated in figure 3.3.

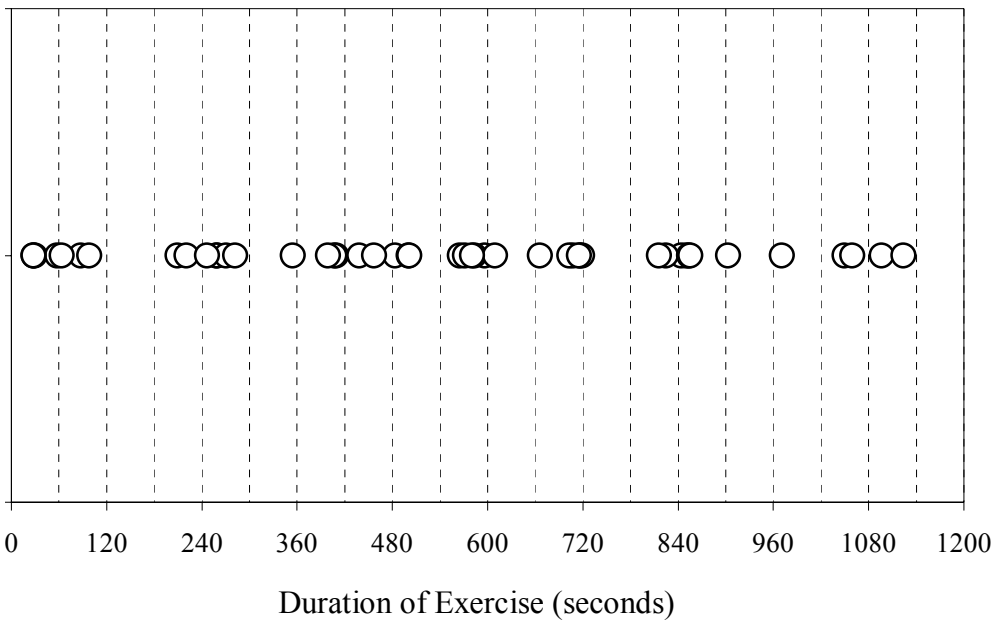


Figure 3.3. Spread of exercise duration using the STEEP protocol.

Co-morbidity and drug therapy

As expected in a study of ischaemic cardiomyopathy, heart failure patients were more likely to have had a previous myocardial infarction (table 3.3). While atrial fibrillation (AF) and type 2 diabetes mellitus were more prevalent in patients with heart failure, the between group differences were not significant.

Mean serum creatinine was higher, and estimated glomerular filtration rate (eGFR) lower, in the HF-cachexia group than in the CAD and healthy control groups (table 3.4). Although sodium, albumin and haemoglobin concentrations were lowest in the HF-cachexia group, the between group differences were not significant.

Patient groups were on appropriate contemporary drug therapy (table 3.5). Although heart failure patients with cachexia were less likely to be receiving a beta-blocker, there was no significant difference in beta-blocker, angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) therapy between the patient groups. As would be anticipated, there was less statin therapy in the IDCM group than in the patient groups with CAD.

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	IDCM (n=7)
Previous MI (%)	70	75	20*	0*
Diabetes (%)	30	45	10	14.3
Hypertension (%)	10	25	30	0
Angiography (%)	60	65	80	0
AF (%)	30	10	10	14.3

Table 3.3. Co-morbidity. MI = myocardial infarction and AF = atrial fibrillation. Comparisons using Fisher's exact test. For HF-cachexia vs each other group: *p<0.05.

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	IDCM (n=7)	HC (n=9)
Creatinine (μmol/l)	127.5 (21.5)	129.1 (29.7)	96.4 (14.2)*	126.6 (24.8)	84.6 (8.2)*
eGFR (ml/min/ 1.73m^2)	46.2 (6.4)	53.4 (13.8)	72 (10.3)**	54.7 (8.6)	74.7 (9.0)**
Sodium (Na^+) (mmol/l)	138.7 (4.7)	141.0 (3.3)	142.1 (1.5)	-	141.3 (1.9)
Albumin (g/l)	39.8 (5.0)	44.0 (3.2)	43.6 (2.4)	-	-
Haemoglobin (g/dl)	12.4 (2.0)	13.8 (1.5)	13.6 (0.8)	-	13.1 (1.0)

Table 3.4. Baseline blood results. eGFR = estimated glomerular filtration rate (Modification of Diet in Renal Disease 1). Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. For HF-cachexia vs each other group: *p<0.05, **p<0.01.

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	IDCM (n=7)
Beta-blocker (%)	40	75	70	85.7
ACE inhibitor (%)	90	85	70	85.7
ARB (%)	10	5	10	0
Loop diuretics (%)	90	80	0†††	85.7
Digoxin (%)	50	20	0*	57.1
Statin (%)	70	80	90	28.6

Table 3.5. Drug therapy. ACE Inhibitor = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker. Comparisons using Fisher's exact test. For HF-cachexia vs each other group: *$p<0.05$, $^{\dagger\dagger\dagger}p<0.0001$.

Body morphology (figure 3.4)

Mean BMI was significantly lower in the HF-cachexia group than in each of the other groups: HF-cachexia 20.0 (5.0) $Kg.m^{-2}$ compared to HF-no cachexia 29.2 (4.7) $Kg.m^{-2}$, CAD 28.8 (3.7) $Kg.m^{-2}$, IDCM 30.5 (4.6) $Kg.m^{-2}$, and HC 29.6 (7.6) $Kg.m^{-2}$ ($p<0.01$ for HF-cachexia compared to each other group).

Mean percentage body fat was also significantly lower in the HF-cachexia group than in each of the other groups: HF-cachexia 15.5 (3.9) % compared to HF-no cachexia 31.7 (6.1) %, CAD 28.5 (5.7) %, and HC 32.6 (10.9) % ($p<0.05$ for HF-cachexia compared to each other group).

Mean waist circumference was lowest in the HF-cachexia group, but this was only significant in comparison to HF-no cachexia group: HF-cachexia 836.2 (119.8) mm, HF-no cachexia 1021.8 (134.6) mm, CAD 977.2 (105.2) mm, and HC 957.8 (150.6) mm ($p<0.01$ for HF-cachexia compared to HF-no cachexia).

Conversely, mean FFMI did not differ between the groups: HF-cachexia 18.0 (3.3) $Kg.m^{-2}$, HF-no cachexia 19.6 (2.1) $Kg.m^{-2}$, CAD 20.7 (2.4) $Kg.m^{-2}$, and HC 19.4 (2.7) $Kg.m^{-2}$.

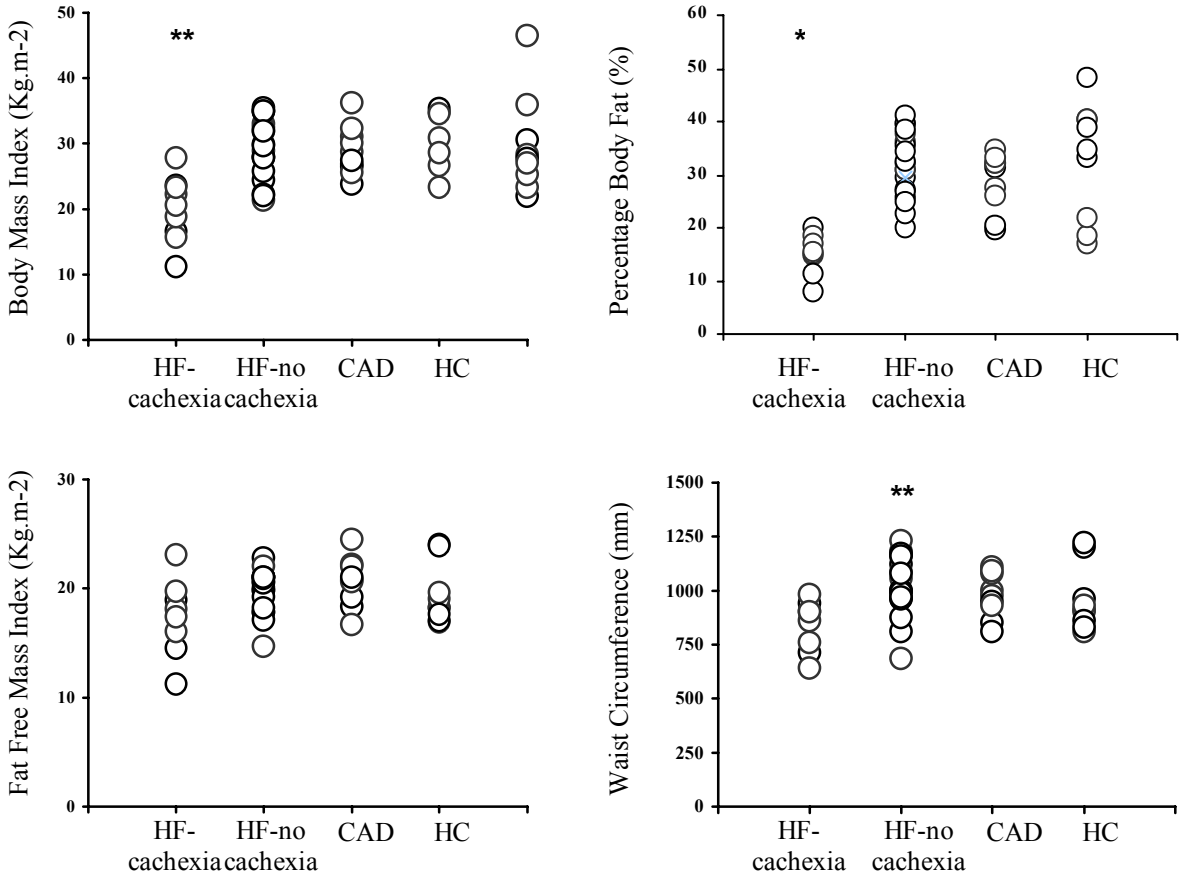


Figure 3.4. Body composition. HF-cachexia, n=10; HF-no cachexia, n=20; CAD, n=10; HC, n=9. Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. For HF-cachexia vs each other group: *p<0.05, **p<0.01.

Serum cytokines and C-reactive protein (CRP) (figure 3.5)

Serum concentrations of TNF-α were highest in patients with IDCM, although not significantly higher than in patients with HF-cachexia: HF-cachexia 27.2 (21.8-132.8) pg/ml, HF-no cachexia 11.7 (2.6-27.3) pg/ml, CAD 25.6 (5.3-51.3) pg/ml, IDCM 42.8 (12.7-85.8) pg/ml, and HC 12.7 (3.6-16.3) pg/ml (Biomarker concentrations given as median (range). TNF-α levels were significantly higher in patients with HF-cachexia than in patients with HF-no cachexia and controls ($p<0.01$ for both comparisons), but not significantly higher than in patients with CAD.

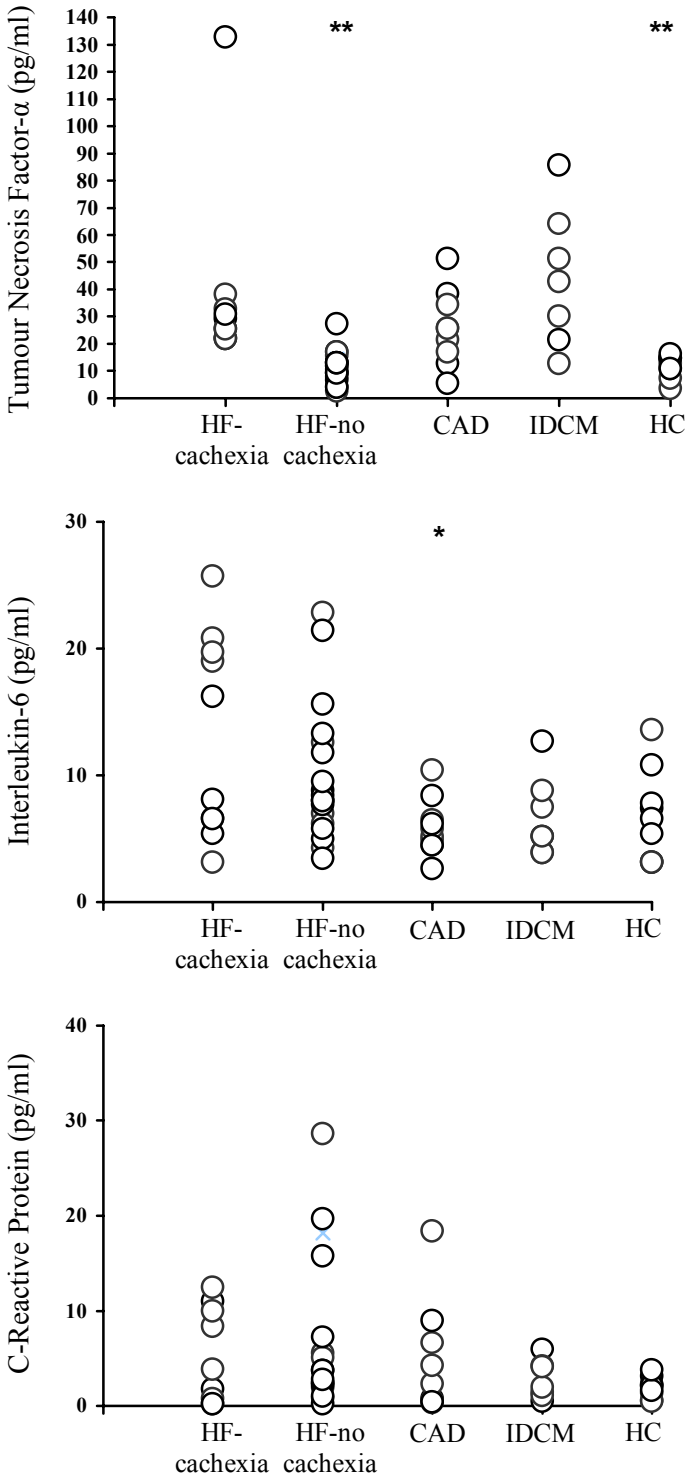


Figure 3.5. Circulating cytokines and C-reactive protein. HF-cachexia, n=10; HF-no cachexia, n=20; CAD, n=10; HC, n=9. Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. For HF-cachexia vs each other group: *p<0.05, **p<0.01.

Serum concentration of IL-6 was highest in patients with HF-cachexia, but this was only significant in comparison to the CAD group: HF-cachexia 12.2 (3.1-25.7) pg/ml, HF-no cachexia 8.2 (3.4-22.8) pg/ml, CAD 5.5 (2.6-10.4) pg/ml, IDCM 5.2 (3.9-12.7) pg/ml, and HC 6.6 (3.1-13.6) pg/ml ($p<0.05$ for HF-cachexia compared to CAD group).

CRP concentration did not differ between the groups: HF-cachexia 3.8 (0.2-12.5) mg/l, HF-no cachexia 3.7 (0.2-28.6) mg/l, CAD 2.3 (0.3-18.4) mg/l, IDCM 2.0 (0.5-6.0) mg/l, and HC 2.0 (0.5-3.7) mg/l.

Skeletal muscle histology

There was no microscopic evidence of muscle inflammation. Quantitative analysis of fibre type composition (percentage area of type I, IIA and IIB fibres), fibre thickness, and muscle area is awaited.

Skeletal muscle cytokine gene expression

There was no between group difference in relative expression of mRNA for TNF-α, IL-1β, IL-6 or IL-18 in skeletal muscle (table 3.6).

	HF-cachexia (n=7)	HF-no cachexia (n=14)	CAD (n=5)	HC (n=5)
TNF-α (relative units)	0.74 (0.70-0.79)	0.72 (0.69-0.81)	0.70 (0.70-0.72)	0.72 (0.70-0.75)
IL-1β (relative units)	0.84 (0.78-1.00)	0.81 (0.77-0.95)	0.83 (0.80-0.94)	0.84 (0.79-0.91)
IL-6 (relative units)	0.96 (0.90-0.98)	0.90 (0.82-1.01)	0.93 (0.89-1.00)	0.96 (0.92-0.98)
IL-18 (relative units)	1.20 (1.04-1.23)	1.12 (0.97-1.26)	1.17 (1.04-1.21)	1.24 (0.95-1.53)

Table 3.6. Relative expression of mRNA for cytokines in skeletal muscle. Cytokine expression given as a ratio relative to β-actin expression. Biomarkers given as median (range). TNF-α = tumour necrosis factor- α; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-18 = interleukin-18. Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. For HF-cachexia vs each other group: $p>0.05$ for all comparisons.

Skeletal muscle CDK inhibitor gene expression

There was no between group difference in relative expression of mRNA for p21, p27 or p57 in skeletal muscle (table 3.7).

	HF-cachexia (n=7)	HF-no cachexia (n=14)	CAD (n=5)	HC (n=5)
p21 (relative units)	1.16 (1.13-1.32)	1.08 (0.98-1.33)	1.24 (1.06-1.30)	1.15 (1.10-1.20)
p27 (relative units)	1.20 (1.09-1.26)	1.21 (1.04-1.32)	1.20 (1.18-1.26)	1.24 (1.16-1.34)
p57 (relative units)	1.09 (1.03-1.15)	1.06 (1.04-1.10)	1.08 (0.90-1.11)	1.08 (0.94-1.12)

Table 3.7. Relative expression of mRNA for CDK inhibitors in skeletal muscle. CDK inhibitor expression given as a ratio relative to β-actin expression. Biomarkers given as median (range). Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. For HF-cachexia vs each other group: $p>0.05$ for all comparisons.

Skeletal muscle cytokine and CDK inhibitor protein expression

The lack of difference in gene expression was confirmed by finding no difference in TNF-α, IL-6 or p21 protein expression in a subgroup of subjects.

Discussion

Using the arbitrary definition of cachexia as an unintentional weight loss of ≥7.5% body weight over ≥6 months we found that the reduction in body weight in cachectic heart failure patients is predominantly due to loss of fat with the relative preservation of fat free mass, contradicting prior hypotheses and assumptions about the nature of cardiac cachexia. We found that heart failure patients with cachexia had significantly lower BMI and percentage body fat than the other subject groups, but with a non-significant reduction in fat free mass index.

In addition to being unable to demonstrate evidence of muscle wasting in our patients with cardiac cachexia, and despite evidence of increased concentrations of cytokines in the blood, there was no local cytokine activation in skeletal muscle. There was an outlying TNF-α concentration in the HF-cachexia group, but even when excluded there remained a clear trend to higher levels in the cachectic patients. There was a spread of TNF-α concentration in the CAD group. A possible explanation for this would be the presence of patients with unstable coronary disease in the CAD group. Unstable CAD is known to be associated with increased systemic inflammation.

While our finding of increased blood cytokines is very consistent with prior studies (Levine *et al*, 1990; McMurray *et al*, 1991; Anker *et al*, 1999), our muscle findings disagree with the only other study to quantify the expression of cytokines in skeletal muscle in heart failure (Gielen *et al*, 2003). Geilin *et al* reported increased relative expression of mRNA for TNF-α, IL-1β and IL-6 in skeletal muscle of similar numbers of patients with heart failure compared to healthy controls (Gielen *et al*,

2003). Importantly, their study population consisted of patients with predominantly idiopathic dilated cardiomyopathy (IDCM). One possible explanation for our discrepant findings is that patients with IDCM have tissue as well as blood cytokine activation compared to patients with ischaemic heart disease. Although the findings from previous studies comparing circulating cytokines in ischaemic and dilated cardiomyopathy have been conflicting, we found higher concentrations of circulating TNF-α in patients with IDCM than in those with ischaemic heart failure (Conraadsa *et al*, 2003; Munger *et al*, 1996; Tentolouris *et al*, 2004). Increased expression of TNF-α has also been reported in the myocardium of patients with IDCM (Satoh *et al,* 2005). This raises the question of whether the skeletal muscle inflammation observed by Geilin *et al* is related to the process causing IDCM rather than arising as a consequence of heart failure *per se*.

Another possible explanation could be differences in drug therapies with supposed anti-inflammatory effects. ACE inhibitor, ARB and beta-blocker therapy have been shown to be associated with reduced circulating cytokines or CRP (Gullestad *et al*, 1999; Gurlek *et al*, 2001; Joynt *et al*, 2004). There was very high use of ACE inhibitor therapy in both the Geilin *et al* and our study (95% and 90%). 40% of heart failure patients in the Geilin *et al* study were treated with beta-blockers in comparison to 75% of our HF-no cachexia group and 40% in our HF-cachexia group. While this difference between our groups should be noted, observational evidence that beta-blocker therapy is associated with reduced CRP in heart failure is not supported by the Metoprolol Controlled-Release Randomised Intervention Trial (MERIT-HF) substudy which reported no effect of beta-blocker therapy on TNF-α or IL-6

concentrations (Joynt *et al*, 2004; Gullestad *et al*, 2001). As expected in a study of ischaemic heart failure, a large proportion of our patients were on statin therapy (70% in the HF-cachexia and 80% in the HF-no cachexia groups). There is no information on statin use in the Geilin *et al* study, although we might anticipate that this would have been lower due to the predominance of patients with non-ischaemic heart failure. Contrary to the possible anti-inflammatoy effect of statins it is important to note that they can also be associated with skeletal muscle myositis in a small proportion of patients.

Of further importance are differences in drug therapies with metabolic effects. Recent studies have demonstrated that beta-blockers, ACE inhibitors and ARBs prevent lipolysis and weight loss (Lainscak *et al*, 2006; Anker *et al*, 2003; Cabassi *et al*, 2006). Two small studies have suggested that beta-blocker therapy, by reducing catecholamine mediated lipolysis, may be associated with increased body fat and weight gain in heart failure, particularly in patients with cachexia (Hryniewicz *et al*, 2003; Lainscak *et al*, 2006). Although there was no significant difference in drug therapy between our HF-cachexia and HF-no cachexia groups the observation that patients with cachexia were less likely to be receiving a beta-blocker may be of importance.

Having found evidence of increased cytokines in the blood of our patients with cardiac cachexia, but no evidence of cytokine activation in skeletal muscle, this raises the question of where the circulating cytokines are coming from. Adipose tissue is known to produce and secrete TNF-α, and it has previously been suggested that

dysregulation of its production could be important in cachexia (Warne *et al*, 2003). Kern *et al* reported increased adipose tissue TNF-α concentration in obesity, and found a positive relationship between TNF-α and both BMI and body fat. Interestingly, very obese subjects were found to have reduced adipose tissue TNF-α (Kern *et al*, 1995).

In addition to finding no local cytokine activation, and contrary to our groups previous findings in patients with chronic obstructive pulmonary disease (COPD), there was no muscle tissue activation of cell cycle inhibitors in our heart failure patients with cachexia. As discussed previously, the definition of cachexia in COPD requires a reduction in FFMI in addition to BMI. This finding could be explained simply by the absence of muscle wasting in our HF-cachexia patients.

In conclusion, weight loss in ischaemic heart failure predominantly involves the loss of adipose tissue with the relative preservation of muscle and bone. Despite the presence of increased circulating levels of inflammatory cytokines we found no evidence of activation of skeletal muscle cytokines or cell cycle inhibitors in our cachectic heart failure patients.

CHAPTER 4

ENERGY METABOLISM IN CARDIAC CACHEXIA

Introduction

Having found that the reduction in body weight in our heart failure patients with cachexia was predominantly due to loss of fat mass, with the relative preservation of fat free mass, we were interested to assess substrate energy metabolism in our study population.

A number of small studies have previously assessed changes in whole body substrate metabolism in heart failure. Increased levels of fat oxidation and free fatty acids, with decreased glucose oxidation and muscle glucose uptake, have consistently been reported. Increased protein turnover has also been reported, but in some studies this has only been present in patients with increased resting energy expenditure (Schneeweiss *et al*, 1987; Lommi *et* al, 1998; Norrelund *et al,* 2006; Toth *et al*, 2006). To the best of our knowledge there are no previous studies comparing substrate metabolism in heart failure patients with and without cachexia.

We hypothesised that the reduction in body fat in our cachectic heart failure patients would be associated with a higher rate of fat oxidation than in our heart failure patients without cachexia.

Methods

Subject groups

As in the previous chapter, five groups of subjects were studied. Three were groups of patients with stable coronary artery disease (CAD): 1) HF-cachexia - patients with CAD, heart failure (HF), reduced left ventricular (LV) systolic function and cardiac cachexia, n=10; 2) HF-no cachexia - those with CAD, HF and reduced LV systolic function but no cachexia, n=20; and 3) CAD - those with CAD but no symptoms of HF and preserved LV systolic function, n=10. The other subject groups were 4) IDCM - patients with dilated cardiomyopathy, n=7; and 5) HC - healthy controls, n=9.

Cardio-respiratory exercise testing and expired gas analysis

All subjects had fasted overnight and performed a symptom limited cardio-respiratory treadmill exercise test using the STEEP (Standardised Exponential Exercise Protocol) protocol (table 2.1) (Northridge et al, 1990). Continuous 12-lead ECG monitoring and heart rate and blood pressure response were recorded. Exhaled air was analysed to determine metabolic gas exchange with a metabolic cart. Oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were determined on-line with breath-by-breath analysis. Resting breath analysis was performed prior to exercise until a steady state was achieved. The peak VO_2 was averaged over the last 30 seconds of exercise. The respiratory exchange ratio (RER) was determined as the ratio of VCO_2/VO_2. Rate of fat and carbohydrate oxidation was calculated by indirect calorimetry using the derived equations of Frayn (Frayn, 1983).

Derived equations for the calculation of substrate oxidation

When glucose is oxidised according to the equation:

$$\text{Glucose } (C_6H_{12}O_6) + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2$$

6 mol of O_2 are consumed and 6 mol of CO_2 are produced for each mole (180 g) of glucose oxidised, and the respiratory quotient ($RQ = VCO_2/VO_2$) is thus 1.00 (table 4.1).

When a typical fat (PSOG, palmitoyl-stearoyl-oleoylglycerol) is oxidised according to the equation:

$$\text{PSOG } (C_{55}H_{104}O_6) + 78\ O_2 \rightarrow 55\ CO_2 + 52\ H_2O$$

78 mol of O_2 are consumed and 55 mol of CO_2 produced for each mole (861 g) of fat oxidised.

Fuel	**O_2 l/g fuel**	**CO_2 l/g fuel**
Glucose	0.746	0.746
Fat	2.03	1.43
Protein	0.966	0.782
	6.04*	4.89*

Table 4.1 Volumes of O_2 consumed and CO_2 produced in oxidation of various fuels. Reproduced from Frayn, 1983. 1 mol of gas occupies 22.4 liters. * Volumes expressed per g urinary nitrogen.

The amount of protein oxidised may be estimated from urinary nitrogen excretion. Most urinary nitrogen (>80%) is in the form of urea with 1 g of urinary nitrogen arising from approximately 6.25 g of protein (Magnus-Levy, 1907).

Therefore, in a subject oxidising c grams of carbohydrate (as glucose) and f grams of fat per minute, and excreting n grams of urinary nitrogen per minute, the total O_2 consumption and total CO_2 production, using values from table 4.1, are thus given by:

$$VO_2 \text{ (l/min)} = 0.746\ c + 2.03\ f + 6.04\ n$$

$$VC0_2 \text{ (l/min)} = 0.746\ c + 1.43\ f + 4.89\ n$$

These equations can be solved for the unknown variables c and f (gaseous exchange and urinary nitrogen excretion being measurable) as follows:

$$c = 4.55\ VCO_2 - 3.21\ VO_2 - 2.87\ n$$

$$f = 1.67\ VO_2 - 1.67\ VCO_2 - 1.92\ n$$

Making the assumption that there is neglible protein oxidation the equations become:

$$\text{CHO oxidation (g/min)} = 4.55\ VCO_2 - 3.21\ VO_2 \text{ (l/min)}$$

$$\text{Fat oxidation (g/min)} = 1.67\ VO_2 - 1.67\ VCO_2 \text{ (l/min)}$$

This standard technique for measuring substrate metabolism in vivo makes a number of assumptions and thus has limitations. Notably, calculated estimates are net substrate oxidation rates (ie. calculated carbohydrate oxidation represents the oxidation of either endogenous glycogen or exogenously administered carbohydrate). In addition, it assumes that there is negligible protein oxidation. While this assumption may have been anticipated to be a significant limitation to this technique in heart failure patients, our finding of preserved fat free mass in our non-cachectic and cachectic patients suggested this is a reasonable assumption to make. It would be ideal to have measured urinary nitrogen. If this assumption is incorrect and our

patients had increased protein turnover, the equations would overestimate rates of oxidation of both substrates, with the error being greater for rate of carbohydrate metabolism.

Results

Expired gas analysis and calculated rates of substrate oxidation

The HF-cachexia group had the highest rate of fat oxidation and the lowest rate of carbohydrate oxidation, although with the small subject numbers the differences were not statistically significant (table 4.1)(figure 4.1).

	HF-cachexia (n=10)	**HF-no cachexia (n=20)**	**CAD (n=10)**	**HC (n=9)**
Resting VO_2 ($mL.min^{-1}$)	297.6 (96.7)	344.6 (106.6)	301.4 (50.8)	283.7 (49.2)
Resting CO_2 ($mL.min^{-1}$)	237.9 (40.3)	296.4 (94.2)	290.9 (79.4)	248.9 (53.8)
Respiratory exchange ratio (RER)	0.77 (0.1)	0.85 (0.1)	0.82 (0.1)	0.88 (0.1)
Fat oxidation (mg/min)	138.6 (112)	91.3 (46.7)	96.0 (45.8)	61.0 (18.4)
CHO oxidation (mg/min)	45.6 (167.2)	233.1 (143.2)	143.8 (127.7)	215.9 (44.6)

Table 4.2. Expired gas analysis and rates of substrate metabolism. Differences between groups assessed by ANOVA. For HF-cachexia vs each other group: $p>0.05$ for all comparisons.

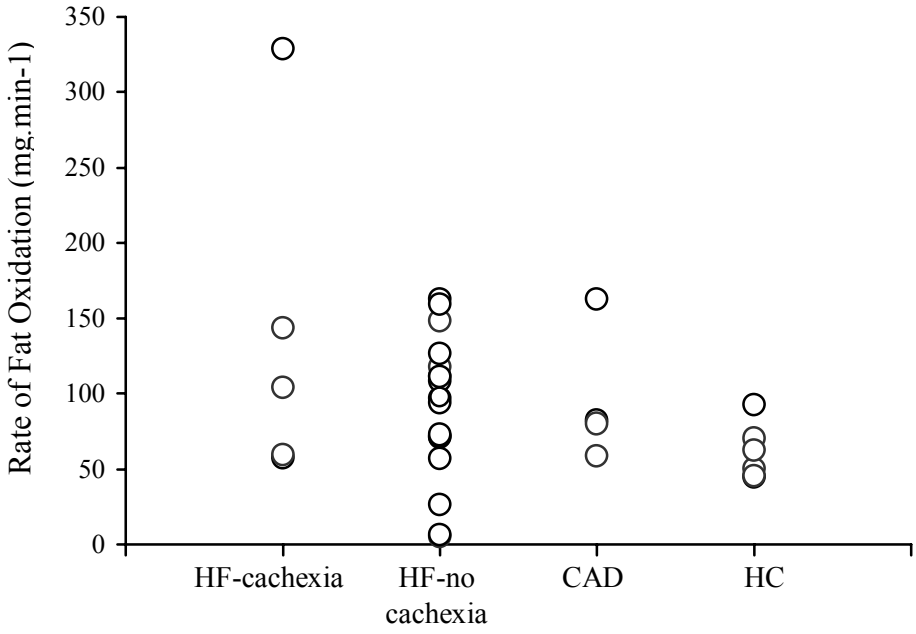


Figure 4.1. Rates of fat oxidation by subject group. Differences between groups assessed by ANOVA. For HF-cachexia vs each other group: $p>0.05$ for all comparisons.

Discussion

We found that our cachectic heart failure patients had altered substrate metabolism, with increased rates of fat oxidation and decreased rates of carbohydrate oxidation, compared to heart failure patients without cachexia (although with our small subject numbers the differences did not achieve statistical significant). This raises the question of what is mediating increased fat metabolism and loss of fat mass in cardiac cachexia.

A key role for the sympathetic nervous system with catecholamine mediated lipolysis has been proposed for many years. Catecholamines, through binding to β-receptors on the surface of adipocytes, and via a cyclic adenosine monophosphate (cAMP) dependent pathway, result in the phosphorylation, and hence activation, of lipase. Studies comparing circulating noradrenaline levels have tended to find higher concentrations in cachectic than non-cachectic heart failure patients (Nagaya *et al*, 2001; Hryniewicz *et al*, 2003). Beta-blocker therapy, while not associated with any significant change in catecholamine levels in non-cachectic heart failure patients, has been associated with a greater reduction in noradrenaline concentration in cachectic compared to non-cachectic heart failure patients (Podbregar *et al*, 2002; Hryniewicz *et al*, 2003). Two small studies in heart failure patients have suggested that beta-blockers prevent weight loss, and may be associated with increased body fat and weight gain, particularly in patients with cachexia (Hryniewicz *et al*, 2003; Lainscak *et al*, 2006). This is supported by a recent in vivo study demonstrating that beta-blockers reduce lipolysis in rats (Cabassi *et al*, 2006). While this all supports a significant contribution of the sympathetic nervous system to increased rates of fat

metabolism and the development of cachexia, there are clearly other important mediators involved.

The presence of higher TNF-α concentrations in patients with cachexia has lead to the suggestion that circulating TNF-α may play a role in tissue wasting. (Levine *et al*, 1990; McMurray *et al*, 1991; Dutka *et al,* 1993; Anker *et al,* 1997). Argiles *et* al proposed that elevated circulating TNF-α is responsible for the metabolic alteration in adipose tissue in cachexia (Argiles *et al*, 1997). TNF-α is also produced by adipose tissue itself with the alternative suggestion that, through the autocrine and paracrine actions of promoting lipolysis and inhibiting lipogenesis, that adipose tissue derived TNF-α, as opposed to circulating TNF-α, mediates the depletion of adipose tissue mass in cachexia (Kern *et al*, 1995; Warne *et al*, 2003).

Adipose tissue is now known, in addition to cytokines, to produce hormones important in the regulation of energy metabolism. There has been particular interest in the more recently discovered leptin and adiponectin. Adiponectin, in particular, is known to have an inverse relationship to body mass index and body fat, and to possibly be regulated by TNF-α. (Maeda *et al,* 1996; Chandran *et al,* 2003; Meier *et al,* 2004; Yamauchi *et al,* 2002; Bruun *et al,* 2003; Wang *et al,* 2006; Degawa-Yamauchi *et al,* 2005; Carey *et al,* 2006). The observation that adiponectin induces weight loss in experimental animal models raises the possibility that it may play an important role in the pathogenesis of cachexia (Fruebis *et al,* 2001; Masaki *et al,* 2003).

The presence and significance of increased circulating levels of natriuretic peptides has been well described in patients with heart failure (Yu *et* al, 1999; Troughton *et al*, 2000; Selvais *et al*, 2000; Berger *et al*, 2002, Lee *et al*, 2002). More recently it has been suggested that natriuretic peptides may also have lipolytic and lipid-mobilising actions (Sengenes *et al,* 2000). These actions of natriuretic peptides appear to be independent of catecholamines and mediated by specific adipocyte membrane receptors, which operate via a cGMP-dependent pathway. Although higher levels of BNP have been described in patients with more advanced heart failure no relationship with cachexia has as yet been investigated.

In conclusion, we found that our cachectic heart failure patients had reduced body fat and increased rates of fat metabolism. We propose that adiponectin and natriuretic peptides may be important mediators of this process, possibly under the regulation of the inflammatory cytokines.

CHAPTER 5

INCREASE IN SERUM ADIPONECTIN CONCENTRATION IN PATIENTS WITH HEART FAILURE AND CACHEXIA: RELATIONSHIP WITH LEPTIN, OTHER CYTOKINES AND B-TYPE NATRIURETIC PEPTIDE

Introduction

Having found that our cachectic heart failure patients had reduced body fat and increased fat metabolism we proceeded to investigate possible mediators of this altered energy metabolism and wasting process.

As discussed briefly in the preceding chapter, there has been previous interest in the hormones that regulate appetite and metabolism in patients with heart failure. In particular, leptin production may be inappropriately low in cachectic heart failure patients (Murdoch *et al,* 1999; Schulze *et al,* 2003). More recently adiponectin, an adipose tissue-specific peptide, has been thought to play an important role in the regulation of energy metabolism having been observed to inversely correlate with body mass index and body fat (Maeda *et al,* 1996; Chandran *et al,* 2003; Meier *et al,* 2004). Obesity is associated with reduced adiponectin levels whereas these are increased in anorexia nervosa (Iwahashi *et al,* 2003; Pannacciulli *et al,* 2003; Delporte *et al,* 2003). In experimental animals, including genetically modified ones, administration of adiponectin reduces weight gain or leads to weight loss (depending on the model), possibly by increasing energy expenditure (Fruebis *et al,* 2001; Yamauchi *et al,* 2002; Masaki *et al,* 2003). Plasma adiponectin concentrations have recently been reported to be increased in patients with heart failure (Kistorp *et al,* 2005; George *et al,* 2006).

As discussed previously, a lipolytic and potential lipid-mobilising effect of natriuretic peptides has been described (Sengenes *et al,* 2000). This is supported by the observation that B-type natriuretic peptide (BNP) correlates inversely with BMI in

heart failure patients (Horwich *et al,* 2006). The actions of BNP appear to be mediated by specific adipocyte membrane receptors, which operate via a cGMP-dependent pathway, and may indirectly stimulate adiponectin production (Sengenes *et al,* 2000). Interestingly, N-terminal pro-BNP (NT-proBNP) has previously been observed to have a positive relationship with plasma adiponectin levels in patients with heart failure (Kistorp *et al,* 2005).

We hypothesised that increased fat metabolism in cachectic heart failure patients is mediated by adiponectin, possibly under the regulation of TNF-α and BNP. We therefore measured serum adiponectin concentrations in our cachectic and non-cachectic heart failure patients, and analysed the relationship of adiponectin with body composition, leptin, inflammatory cytokines, BNP and rate of fat oxidation.

Methods

Subject groups

We studied four of our subjects groups. Three were groups of patients with stable coronary artery disease (CAD): 1) HF-cachexia - patients with CAD, heart failure (HF), reduced left ventricular (LV) systolic function and cardiac cachexia, n=10; 2) HF-no cachexia - those with CAD, HF and reduced LV systolic function but no cachexia, n=20; and 3) CAD - those with CAD but no symptoms of HF and preserved LV systolic function, n=10. The fourth group were 4) HC - healthy controls, n=7.

It was important that only patients with CAD were studied as there may be a difference in adiponectin levels between individuals with and without CAD. Two healthy control subjects were excluded for age-matching to the patient groups. Subject groups characteristics and body composition data is therefore revised with this alteration.

Subject characterisation

Subject characterisation by NYHA classification, LVEF, peak VO_2, body morphology and composition, co-morbidity and drug therapy has been described in detail earlier.

Rate of fat oxidation was calculated as described in detail in chapter 4.

Blood sample collection and analysis

All subjects had fasting blood samples taken between 9 and 10 am. Blood samples were placed on ice, allowed to clot for 20 minutes and then centrifuged at 3000 rpm for 15 minutes at 4 °C. The serum or plasma was aliquoted and stored at –80 °C until analysis. Serum adiponectin, leptin, tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) were measured in triplicate using commercially available enzyme-linked immunosorbent assay kits (Quantikines, R&D systems). Cholesterol, triglycerides, VLDL cholesterol, LDL cholesterol, HDL cholesterol and high sensitivity CRP concentrations were also measure using a Hitachi 917 analyser (Roche kits). Fasting glucose and insulin were measured and the homeostasis model assessment (HOMA-IR) index calculated to estimate insulin resistance (Matthews *et al,* 1985). Blood for the measurement of plasma B-type natriuretic peptide (BNP) was added to a chilled tube containing ethylenediaminetetraacetic acid (EDTA). Plasma BNP was measured using the Shionoria immunoradiometric kit (Schering CIS, West Sussex, England).

Statistical analysis

Results are expressed as mean (SD) for baseline characteristics and median (range) for biomarkers. Two-sided tests of significance were used. Normality testing and Bartlett's test for homogeneity of variance were performed and differences between groups assessed by one-way analysis of variance (ANOVA) or Kruskal-Wallis non-parametric ANOVA. Where differences were identified, a Dunnett's or Dunn's multiple comparison *post hoc* test was performed. Fisher's exact test was used to compare categorical characteristics. The data was logarithmically transformed for

linear regression analysis and a Run's test used for departure from linearity. The relationship of adiponectin to other biomarkers was further assessed by multiple linear regression analysis adjusting for age, BMI, eGFR and insulin resistance. Statistical significance was taken at the 5% level.

Results

Subject characteristics

Mean age was higher in the heart failure patients with cachexia than in the other groups, although this difference was not significant. Mean LVEF, peak VO_2 and eGFR were all lower in patients with heart failure than in the control groups (table 5.1 and 5.2). Patients with cachectic heart failure had worse NYHA functional class and a lower peak VO_2 than non-cachectic heart failure patients. Drug therapy for each of the patients groups was previously outlined in table 3.4.

	HF-cachexia (n=10)	**HF-no cachexia (n=20)**	**CAD (n=10)**	**HC (n=7)**
Mean age (years)	72.7	67.6	64.4	63.0
Gender (M/F)	8/2	18/2	9/1	4/3
NHYA (I/II/III)	0/7/3	12/8/0	-	-
LVEF (%)	27.1 (11.9)	35.4 (9.7)	73.0 (6.4)**	68.4 (6.1)**
Peak VO_2 ($ml.Kg^{-1}.min^{-1}$)	9.8 (2.3)	13.6 (3.9)*	21.2 (2.0)**	26.4 (5.5)**

Table 5.1. Subject characteristics. LVEF measured using dual region of interest (normal ≥55%). Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. Categorical characteristics compared using Fisher's exact test. For HF-cachexia vs each other group: *p<0.05, **p<0.01.

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	HC (n=7)
Previous MI (%)	70	75	20*	-
Diabetes (%)	30	45	10	-
Hypertension (%)	10	25	30	-
Mean creatinine (μmol/l)	127.5 (21.5)	129.1 (29.7)	96.4 (14.2)*	85.1 (8.6)*
eGFR (ml/min/1.73m^2)	46.2 (6.4)	53.4 (13.8)	72 (10.3)**	75.9 (8.4)**

Table 5.2. Co-morbidity. eGFR = estimated glomerular filtration rate (Modification of Diet in Renal Disease 1). Categorical variables compared using Fisher's exact test. Differences between groups for continuous variables assessed by ANOVA. *Post hoc* analysis with Dunnett's multiple comparisons. For HF-cachexia vs each other group: *$p<0.05$, **$p<0.01$.

Body composition analysis

Mean body mass index (BMI) and percentage body fat were substantially lower in the heart failure patients with cachexia than in the other groups (table 5.3). HF-cachexia patients had the lowest waist circumference, but this was only significantly lower than in the HF-no cachexia group. There was no significant difference in body composition between the HF-no cachexia, CAD and healthy control groups.

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	HC (n=7)
Body Mass Index (Kg m^{-2})	20.0 (5.0)	29.2 (4.7)**	28.8 (3.7)**	27.0 (4.6)**
Body fat (%)	15.5 (3.9)	31.7 (6.1)**	28.5 (5.7)**	29.2 (9.8)**
Waist circumference (mm)	836.2 (119.8)	1021.8 (134.6)**	977.2 (105.2)	923 (138)

Table 5.3. Body composition. Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. For HF-cachexia vs each other group: *p<0.05, **p<0.01.

Serum adiponectin concentration

The HF-cachexia group had a significantly higher median serum adiponectin concentration than those in each of the other groups (table 5.4) (figure 5.1). This remained true after adiponectin concentration was adjusted for fat mass or BMI. Because adiponectin shows sexual dimorphism (with higher serum concentrations in females), we also repeated this analysis in male subjects only. Adiponectin levels remained significantly higher in HF patients with cachexia: 22.5 (10.2-37.2) μg/ml compared to 7.1 (0.5-16.6) μg/ml in the HF no-cachexia group and 7.0 (0.4-10.7) μg/ml in the CAD controls and 9.8 (2.5-16.8) μg/ml in the healthy controls.

	HF-cachexia (n=10)	HF-no cachexia (n=20)	CAD (n=10)	HC (n=7)
Adiponectin (μg/ml)	23.8 (10.2-37.2)	8.1 (0.5-16.6)*	7.1 (0.4-13.5)*	8.7 (2.5-16.8)*
Adiponectin adjusted for body fat (μg/ml/Kg)	2.2 (0.7-8.4)	0.3 (0.02-0.9)*	0.3 (0.08-0.9)*	0.4 (0.06-1.3)
Adiponectin adjusted for BMI (μg/ml/Kgm^{-2})	1.1 (0.4-3.3)	0.2 (0.02-0.6)*	0.2 (0.01-0.5)*	0.3 (0.07-0.8)*

Table 5.4. Serum adiponectin concentration. Results are expressed as median (range). Differences between groups assessed by ANOVA with *post hoc* analysis with Dunnett's multiple comparisons. For HF-cachexia vs each other group: *p<0.05.

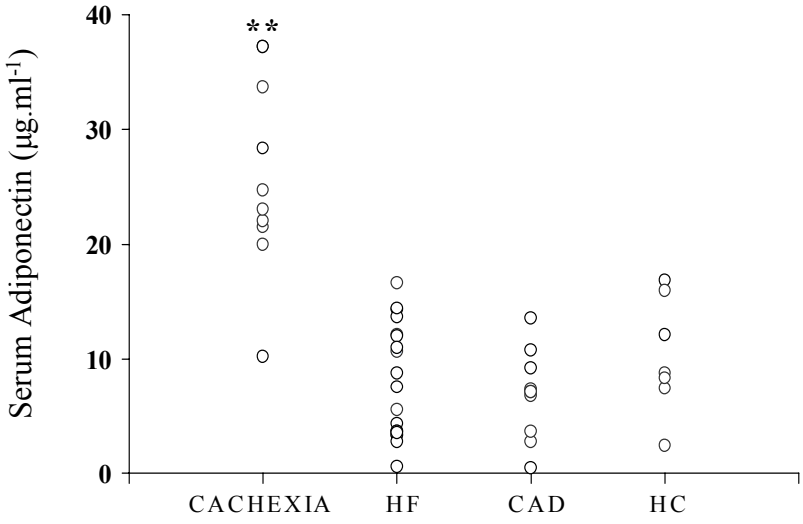


Figure 5.1. Serum adiponectin concentrations in subject groups.

**p<0.05 for cachexia vs each other group

Serum leptin concentration

Patients in the HF-cachexia group had the lowest median serum leptin concentration, although there was no statistically significant difference from the other groups, with the exception of the CAD control group (table 5.5). This difference in leptin was no longer significant after adjusting for fat mass or BMI.

	HF-cachexia (n=10)	**HF-no cachexia (n=20)**	**CAD (n=10)**	**HC (n=7)**
Leptin (ng/ml)	9.5 (2.1-14.0)	12.6 (3.4-55.4)	20.8 (12.4-26.3)**	11.1 (1.5-18.4)
Leptin adjusted for body fat (ng/ml/Kg)	1.0 (0.2-2.8)	0.5 (0.2-1.7)	0.8 (0.7-1.3)	0.4 (0.1-0.8)
Leptin adjusted for BMI ($ng/ml/Kgm^{-2}$).	0.6 (0.1-0.8)	0.4 (0.1-1.6)	0.7 (0.5-0.9)	0.4 (0.1-0.7)

Table 5.5. Serum leptin concentration. Results are expressed as median (range). Differences between groups assessed by ANOVA with *post hoc* analysis with Dunnett's multiple comparisons. For HF-cachexia vs each other group: **$p<0.01$.

Concentrations of other serum cytokines

Serum TNF-α concentration was significantly higher in the HF patients with cachexia than in the HF patients without cachexia and the healthy controls: HF-cachexia 27.2 (21.8-132.8) pg/ml, HF-no cachexia 11.7 (2.6-27.3) pg/ml, CAD 25.6 (5.3-51.3) pg/ml, and HC 10.8 (3.6-16.3) pg/ml ($p<0.01$ for HF-cachexia compared to HF-no cachexia and HC groups). Although TNF-α concentration was higher in HF-cachexia group than in the CAD group, the difference was not statistically significant (figure

5.2). Although IL-6 concentration was higher in HF-cachexia group than in all other groups this was only significant on comparison to the CAD group (figure 5.2): HF-cachexia 12.2 (3.1-25.7) pg/ml, HF-no cachexia 8.2 (3.4-22.8) pg/ml, CAD 5.5 (2.6-10.4) pg/ml, and HC 5.4 (3.1-13.6) pg/ml ($p<0.05$ for HF-cachexia compared to CAD group).

C-reactive protein (CRP)

There was no significant difference in CRP concentration between the groups (figure 5.2): HF-cachexia 3.8 (0.2-12.5) mg/l, HF-no cachexia 3.7 (0.2-28.6) mg/l, CAD 2.3 (0.3-18.4) mg/l, and HC 1.6 (0.5-3.7) mg/l.

B-type natriuretic peptide (BNP)

Patients in the HF-cachexia group had higher median plasma BNP concentration than the HF-no cachexia group, although this difference was not statistically significant: HF-cachexia 484 (79-1609.5) pg/ml, HF-no cachexia 151 (22.5-557) pg/ml, CAD 58.5 (3.0-173.7) pg/ml, and HC 22.5 (5.5-72.0) pg/ml. BNP concentrations were significantly higher in the HF-cachexia group than in the CAD and healthy control groups ($p<0.01$ for both comparisons) (figure 5.2).

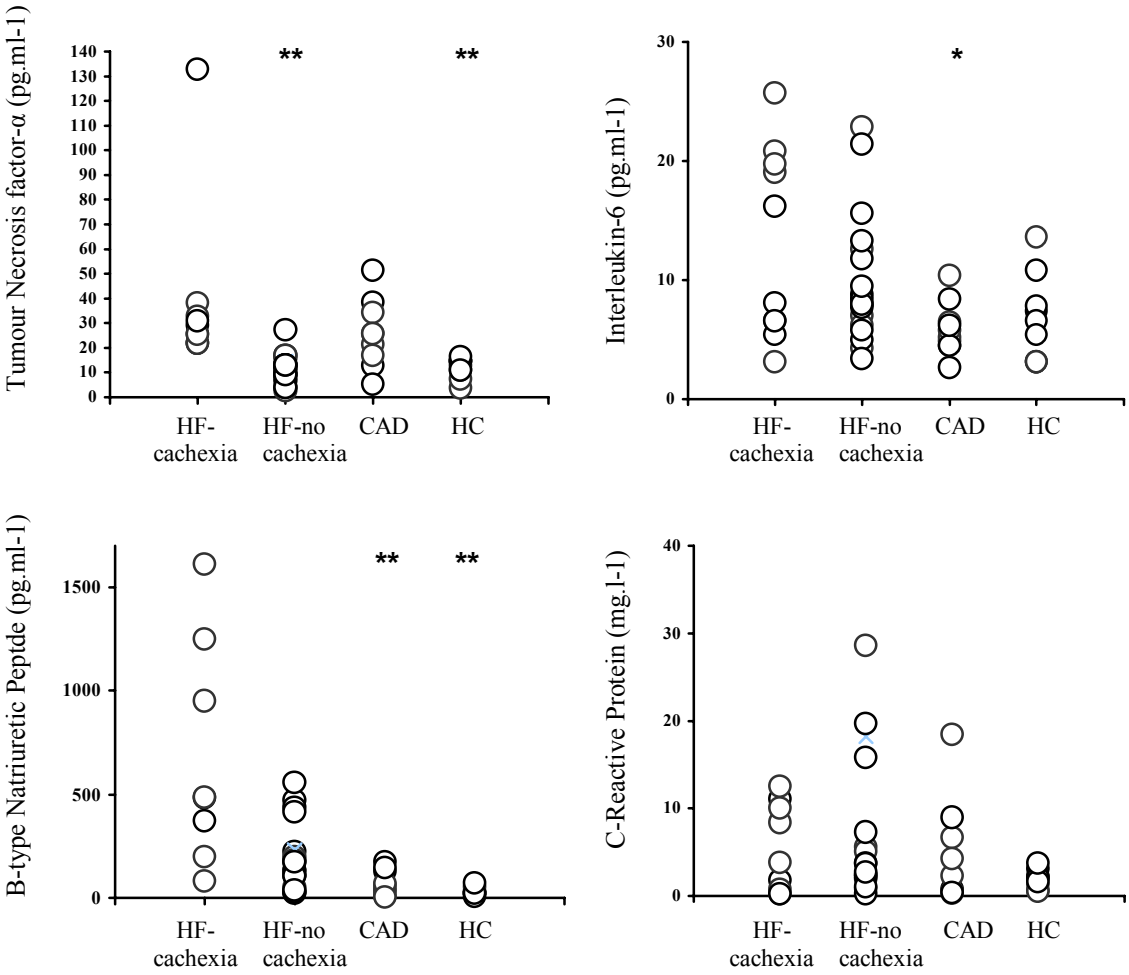


Figure 5.2. Cytokines, B-type natriuretic peptide and C-reactive protein. HF-cachexia, n=10; HF-no cachexia, n=20; CAD, n=10; HC, n=7. Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. *Post hoc* analysis with Dunnett's or Dunn's multiple comparisons. For HF-cachexia vs each other group: *p<0.05; **p<0.01.

Lipid profile

There was no significant difference in the lipid profile of patients with HF and cachexia than the other patient groups (table 5.6).

	HF-cachexia (n=10)	**HF-no cachexia (n=20)**	**CAD (n=10)**	**HC (n=7)**
Cholesterol (mmol/l)	3.4 (2.2-5.5)	3.8 (2.6-5.4)	3.7 (3.1-5.1)	4.5 (3.2-6.4)
Triglyceride (mmol/l)	1.2 (0.8-4.9)	1.8 (0.9-5.0)	1.2 (0.7-2.0)	0.7 (0.6-1.4)
VLDL-Cholesterol (mmol/l)	0.5 (0.4-2.3)	0.8 (0.4-2.3)	0.5 (0.3-0.9)	0.3 (0.3-0.6)
LDL-Cholesterol (mmol/l)	1.4 (0.9-3.3)	2.1 (0.9-3.6)	2.0 (1.2-3.1)	2.6 (1.6-4.2)
HDL-Cholesterol (mmol/l)	0.8 (0.5-1.6)	0.8 (0.6-1.5)	1.1 (0.9-1.6)	1.3 (1.3-2.2)

Table 5.6. Lipid profile. Results expressed as median (range). Differences between groups assessed by ANOVA or Kruskal-Wallis ANOVA. For HF-cachexia vs each other group: $p>0.05$ for all comparisons.

Insulin resistance

The median HOMA-IR index was 1.3 (0.5-33.1) in the HF-cachexia group, 5.9 (0.7-24.8) in the HF-no cachexia group, 2.2 (0.7-7.3) in the CAD control group, and 1.5 (0.7-3.7) in the healthy control group. There was no statistically significantly difference between the groups.

Rate of fat oxidation

Rates of fat oxidation in each subject group are given in table 4.1 and illustrated in figure 4.2.

Correlations

In all patients studied (excluding healthy controls), BMI, percentage body fat, and waist circumference negatively correlated with adiponectin ($r = -0.47$, $r = -0.47$, and $r = -0.44$, respectively; $p<0.01$ for all relationships) (figure 5.3), and positively correlated with leptin concentration ($r = 0.56$, $r = 0.52$, and $r = 0.52$, respectively; $p\leq0.003$ for all relationships).

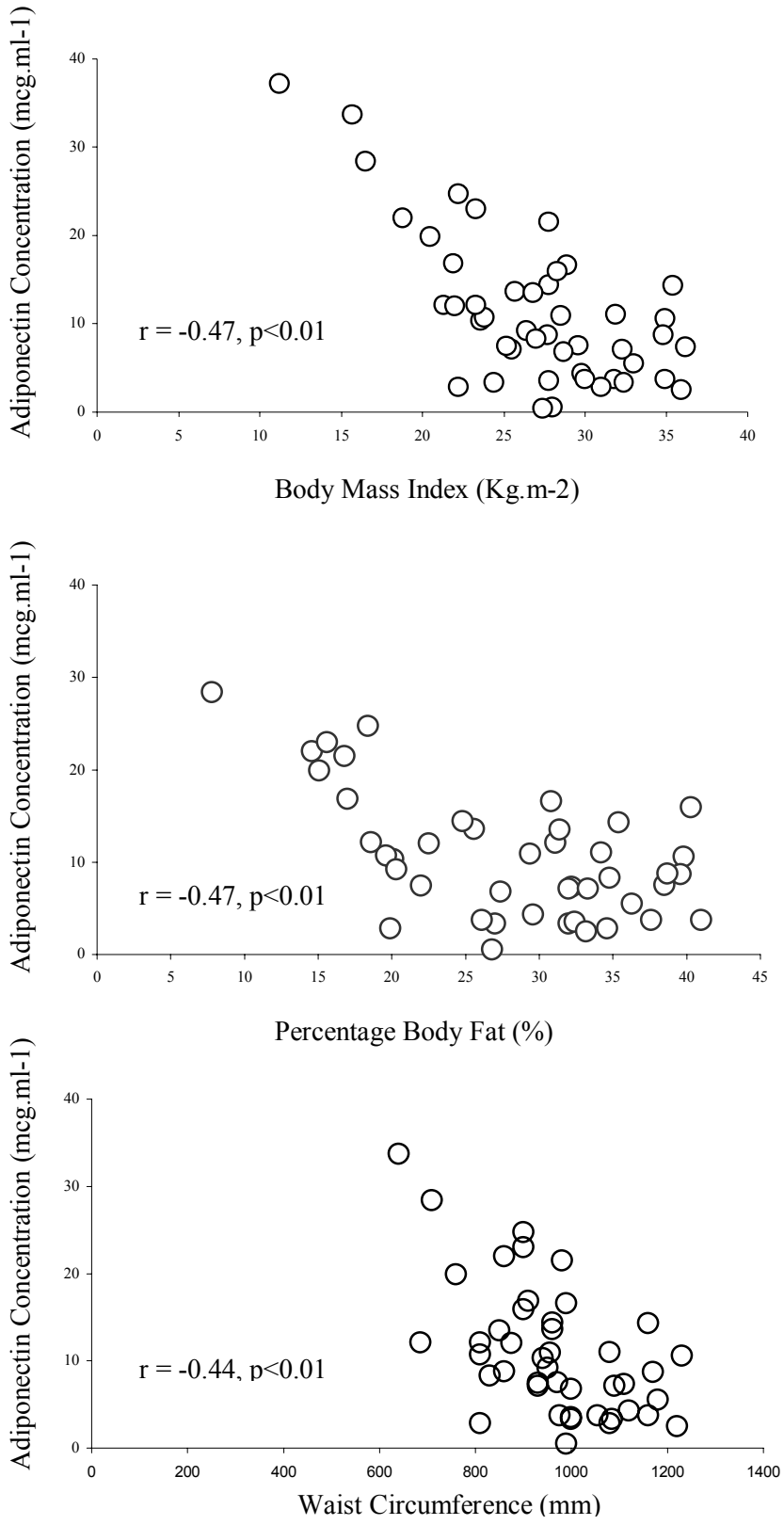


Figure 5.3. Relationship between serum adiponectin and body composition.

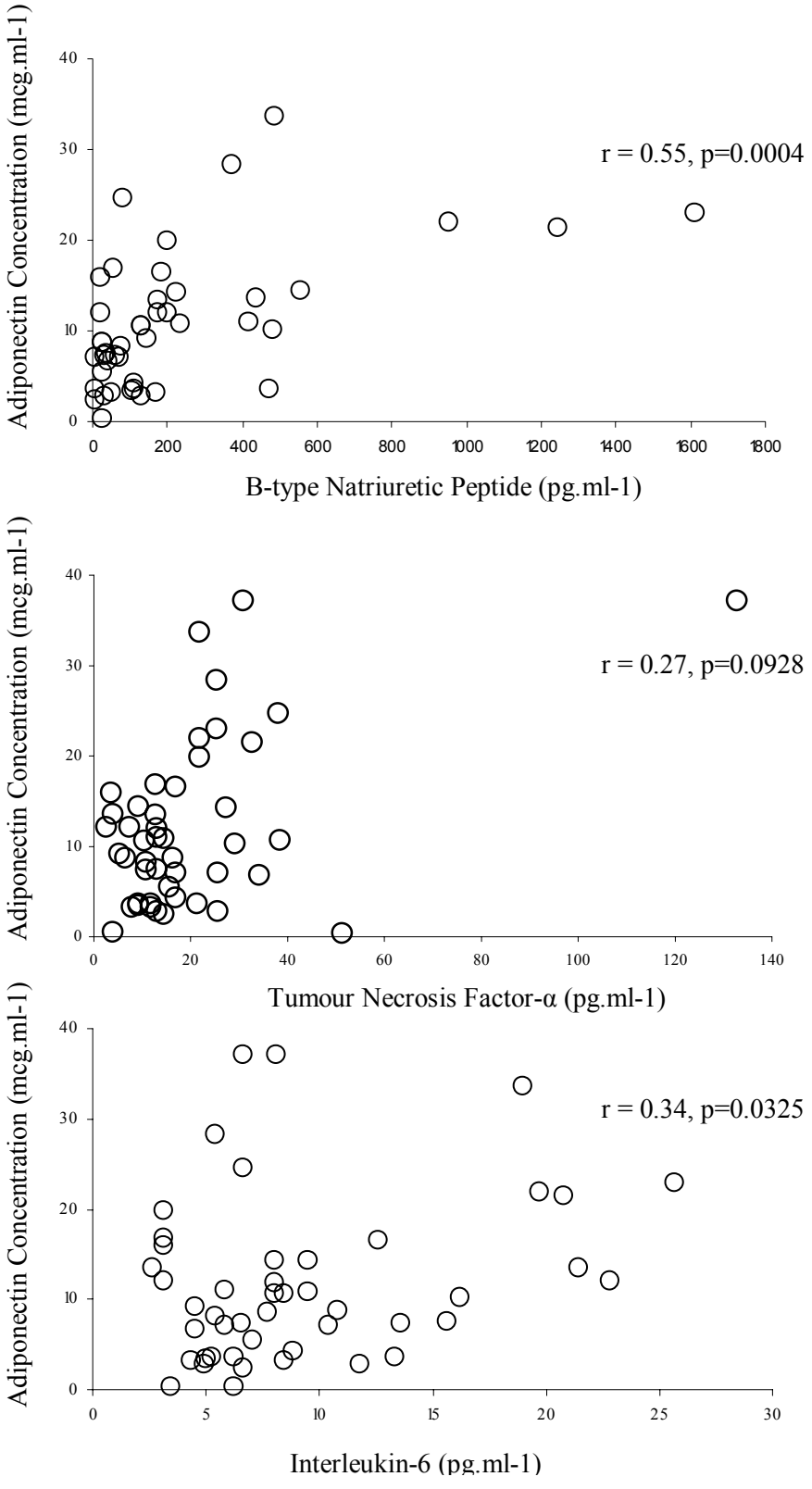


Figure 5.4. Relationship between serum adiponectin, plasma BNP, and inflammatory cytokines.

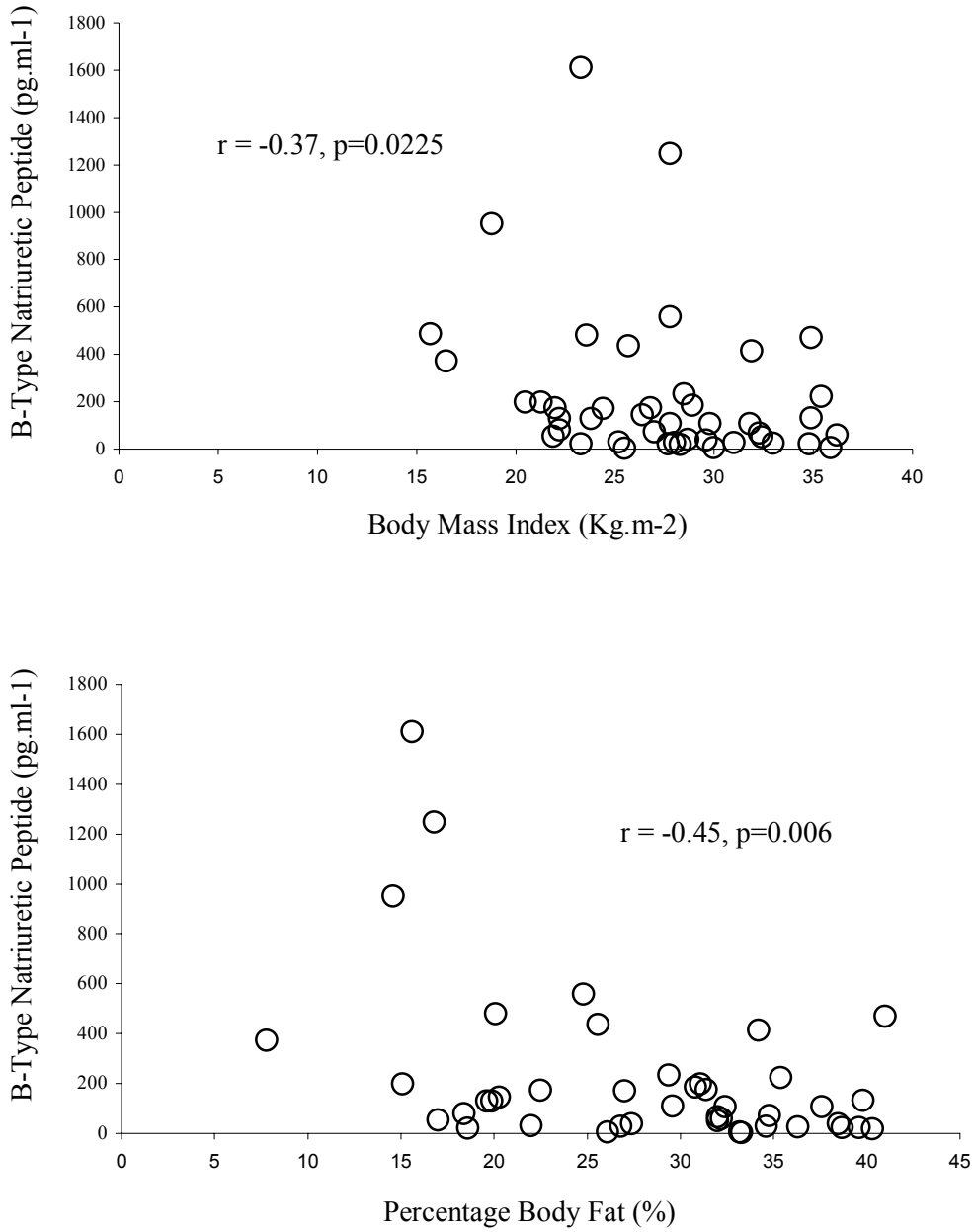


Figure 5.5. Relationship between plasma BNP and body composition.

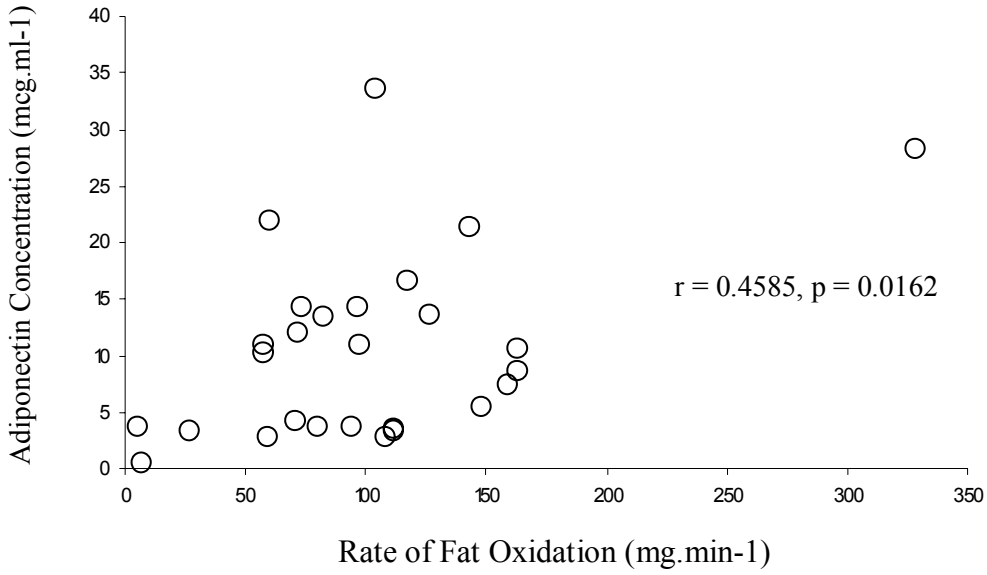
BNP positively correlated with adiponectin ($r = 0.55$, $p = 0.0004$) (figure 5.4) and negatively correlated with leptin ($r = -0.36$, $p = 0.0363$). There was an inverse correlation of BNP with BMI ($r = -0.37$, $p = 0.0225$) and percentage body fat ($r = -0.45$, $p = 0.006$) (figure 5.5).

There was an inverse relationship between adiponectin and insulin resistance ($r = -0.43$, $p = 0.0077$).

Adiponectin positively correlated with IL-6 ($r = 0.34$, $p = 0.0325$) while no relationship was observed with TNF-α ($r = 0.27$, $p = 0.0928$; Runs test $p < 0.05$ with significant departure from linearity) (figure 5.4).

Multiple linear regression analysis, using a model adjusting for age, BMI, eGFR and insulin resistance, demonstrated a positive relationship between adiponectin and BNP ($r = 0.57$, $p = 0.0017$) and TNF-α ($r = 0.41$, $p = 0.0313$).

Rate of fat oxidation positively correlated with adiponectin ($r = 0.57$, $p = 0.0018$) but there was no relationship with BNP ($r = 0.15$, $p = 0.4288$) (figure 5.6).



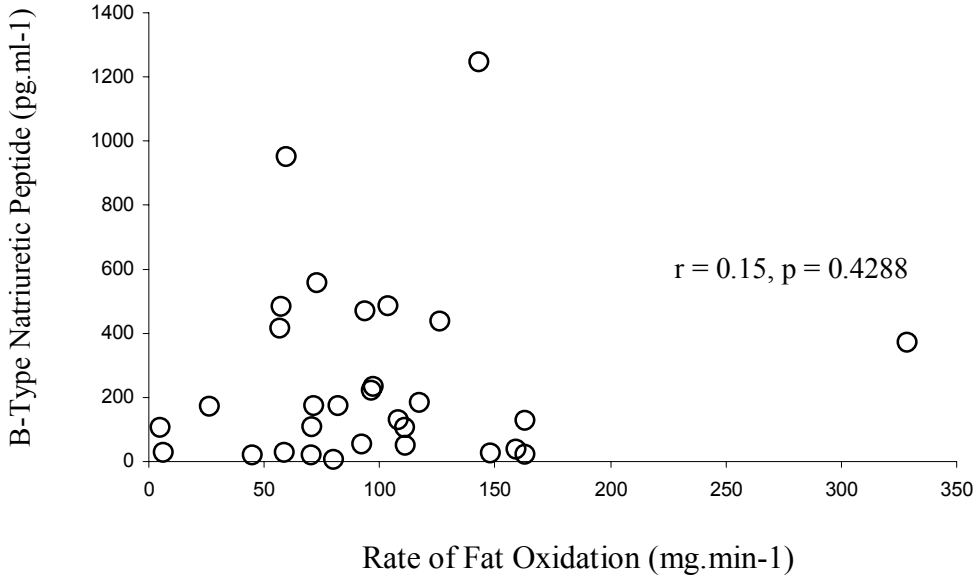


Figure 5.6. Relationship between rate of fat oxidation, serum adiponectin and plasma BNP.

Discussion

We found that our cachectic heart failure patients, with reduced body fat and increased fat metabolism, had markedly elevated serum adiponectin concentrations compared to heart failure patients without cachexia.

This is also to our knowledge the first study to report higher plasma concentrations of BNP in heart failure patients with cachexia (although with our small subject numbers this did not achieve statistical significance).

Furthermore, we observed a positive relationship between adiponectin and BNP. Both negatively correlated with BMI and percentage body fat, and positively correlated with serum TNF-α concentration. While rate of fat oxidation positively correlated with adiponectin concentration, there was no relationship observed with BNP.

At the very least, these findings suggest preservation of the supposed physiological, inverse, relationship between adiponectin and fat mass in cachexia (Chandran *et al,* 2003; Meier *et al,* 2004). More interestingly, our findings raise the possibility that adiponectin, possibly under the influence of TNF-α and BNP, contributes to increased fat metabolism, loss of fat mass and the development of cachexia in heart failure.

Adiponectin levels have been inversely correlated with body mass and fat in the disordered metabolic states of obesity and anorexia nervosa. Adiponectin concentrations are decreased in obesity, while weight loss in obese subjects, induced by either dieting or gastric surgery, is associated with an increase in adiponectin

concentrations (Chandran *et al,* 2003; Meier *et al,* 2004; Holdstock *et al,* 2003). Contrary to this however are recent studies in non-obese, healthy volunteers showing weight loss to be associated with no change or a fall in plasma adiponectin concentrations (Wolfe *et al,* 2004; Mousavinasab *et al,* 2005). Thus, the relationship between change in weight and adiponectin may not be the same in healthy and obese individuals. From our observations, it appears that the relationship of adiponectin to weight loss in cardiac cachexia is also different to that in healthy subjects.

In fact the directional change in serum adiponectin concentrations in cardiac cachexia appears similar to that in patients with anorexia nervosa. Importantly, this eating disorder involves starvation, which is not a feature of cachexia, and neuropsychiatric abnormalities, which may alter endocrine function. Furthermore, the increases in adiponectin reported in anorexia nervosa have been much less marked than we found in cardiac cachexia (Iwahashi *et al,* 2003; Pannacciulli *et al,* 2003; Delporte *et al* 2003).

Obviously, a more direct way of establishing a "cause and effect" relationship between increased adiponectin and reduced weight would be to examine change in weight following administration of the peptide. This has not been done in humans. However, studies conducted in experimental animals have shown a consistent effect of adiponectin to either prevent weight gain or induce weight loss (Fruebis *et al,* 2001; Yamauchi *et al,* 2002; Masaki *et al,* 2003).

What are the possible mechanisms for the increased serum adiponectin concentrations in patients with cachectic heart failure? The factors controlling adiponectin secretion are still poorly understood and controversial. It is striking that our cachectic patients had much higher levels despite a greatly reduced "endocrine" mass (i.e. fat tissue).

TNF-α has been suggested to increase adiponectin secretion, although the literature is conflicting (Bruun *et al,* 2003; Wang *et al,* 2006; Degawa-Yamauchi *et al,* 2005; Carey *et al,* 2006). We found elevated serum TNF-α levels in our cachectic heart failure patients, as shown previously, and a positive relationship with adiponectin concentration (Anker *et al,* 1997; McMurray *et al,* 1991; Sengenes *et al,* 2000). These findings could also be interpreted as being consistent with the proposed anti-inflammatory role of adiponectin.

We also found increased BNP levels in our cachectic heart failure patients, and a positive relationship with adiponectin concentration, raising the possibility that natriuretic peptides might promote adiponectin secretion. As discussed earlier, BNP has lipolytic and potential lipid-mobilising effects, and is thought to indirectly stimulate adiponectin production, acting via adipocyte membrane receptors and a cGMP-dependent pathway (Sengenes *et al,* 2000). Our findings are consistent with Kistorp *et al* who found a positive relationship (albeit of lesser magnitude than seen in the present study) between plasma adiponectin levels and NT-proBNP concentration (Kistorp *et al,* 2005). BNP has previously been demonstrated to correlate inversely with BMI (Horwich *et al,* 2006). In addition to observing a similar

inverse relationship of BNP with BMI in our study, we also found an inverse relationship between BNP and percentage body fat.

Reduced renal clearance, rather than increased secretion, might also account for our findings in cachectic heart failure patients, since adiponectin is cleared from the circulation by the kidneys (Chandran *et al,* 2003; Meier *et al,* 2004). While many of our patients had reduced renal function, as evidenced by a reduced glomerular filtration rate, there was no marked difference in renal function between cachectic and non-cachectic patients. Thus, renal dysfunction is unlikely to explain the higher adiponectin concentrations in the cachectic heart failure patients.

Type 2 diabetes is associated with reduced plasma adiponectin concentration, with the reduction in adiponectin closely related to the degree of insulin resistance and hyperinsulinaemia (Hotta *et al*, 2000; Weyer *et al*, 2001). It was therefore in some ways surprising to find that adiponectin was increased in our patients as heart failure is known to be a state of insulin resistance (Opie *et al,* 2004).

Although not achieving statistical significance with our small subject numbers, as previously described, CRP concentration was highest in patients with heart failure, and elevated in patients with CAD in comparison to healthy subjects (Yin *et al*, 2004; Chirinos *et al*, 2003; Rosenson *et al*, 2004; Arroyo-Espliguero et al, 2004).

The lipid profiles were strikingly similar in the patient groups, achieving currently recommended targets for CAD, with high levels of statin use. With heart failure

patients excluded from the large, randomised, clinical trials, and with the recent observation that reduced lipid levels may be associated with an adverse outcome in chronic heart failure, the efficacy and safety of statin therapy in heart failure patients is currently being investigated by the CORONA study (Rauchhaus *et al*, 2003; Kjekshus *et al*, 2005; Scandinavian Simvastatin Survival Study Group, 1994; Longterm Intervention with Pravastatin in Ischaemic Disease Study Group, 1998; Heart Protection Collaborative Group, 2002).

In conclusion, our cachectic heart failure patients had significantly increased serum concentrations of adiponectin. The observed relationships with BMI, percentage body fat, rate of fat metabolism, serum TNF-α, and plasma BNP concentrations suggests that adiponectin may be a key mediator of the cachectic process, possibly under the influence of TNF-α and BNP.

CHAPTER 6

RELATIONSHIP BETWEEN PLASMA ADIPONECTIN CONCENTRATION AND RATE OF FAT METABOLISM

Introduction

Having found markedly elevated adiponectin levels, an increased rate of fat metabolism, and a positive relationship between the two in our cachectic heart failure patients, we proposed that adiponectin may be a key mediator in the loss of adipose tissue and the development of cardiac cachexia.

A positive association between adiponectin gene expression and lipolytic activity in the adipose tissue of healthy and obese women has previously been observed (Bullo et al, 2005). Furthermore, adiponectin has been shown in animal and in vitro models to stimulate fatty acid uptake and oxidation in skeletal muscle. (Fruebis et al, 2001; Chen et al, 2005; Bruce et al, 2005).

To develop our understanding of the relationship between adiponectin and fat metabolism we proceeded to investigate this further in a population of young healthy male subjects, using an exercise protocol specifically designed to maximise fat oxidation.

Substrate energy metabolism during exercise is determined by the nature, intensity and duration of the activity being undertaken. During low-intensity exercise, at less than 50% peak oxygen consumption (peak VO_2), fat is the predominant fuel, accounting for more than half of the total energy production. At 60–65% peak VO_2 fat and carbohydrate contribute equally, and at higher intensity, 70-75% peak VO_2, muscle glycogen is the major fuel (Romijn *et al*, 1993). Resistance exercise, through activation of growth hormone, is known to further promote fat oxidation (Bamman *et*

al, 2001; Nindl *et al*, 2001). Previous reports of the response of adiponectin to exercise in healthy subjects have been conflicting. Various programs of exercise intensity and duration have been used, and while all have demonstrated significant increases in rates of fat metabolism most have reported no change in adiponectin concentration (Hulver et al, 2002; Yatagai et al, 2003; Ferguson et al, 2004; Jurimae et al, 2005; Ring-Dimitriou et al, 2006; Klimcakova et al, 2006; Brooks et al, 2006; Bobbert et al, 2007).

We hypothesised that there may be a causal relationship between adiponectin concentration and rate of fat metabolism. To investigate this further we measured rate of fat oxidation and plasma adiponectin concentration in healthy male subjects during an exercise program, of resistance and prolonged, low intensity exercise, specifically designed to maximise fat metabolism.

Methods

As part of an ongoing interest in our group in substrate energy metabolism during exercise, we designed an exercise protocol to maximise rate of fat metabolism. We proceeded to further investigate the relationship between adiponectin and rate of fat oxidation using this protocol in young healthy male subjects.

Subjects

We recruited eleven healthy male subjects with a mean age of 25 (4.2) years. The study was approved by the local ethics committee. Written informed consent was obtained from all participants.

Body morphology

All subjects had height, weight and body mass index measured. Skinfold thickness was measured with fat mass and fat free mass determined using the method described by Durnin and Womersley (Durnin et al, 1974). Harpenden callipers were used and skinfold thickness measured at four sites on the right hand side of the body (triceps, biceps, subscapular and suprailiac crest).

Treadmill exercise testing

Subjects attended on three separate occasions to perform a treadmill exercise test with respiratory gas analysis.

At visit 1, peak oxygen consumption (peak VO_2) was measured during uphill walking at a constant speed using a modification of the Taylor treadmill test (Taylor et al,

1955). Expired air samples were collected using Douglas bags, with oxygen consumption (VO_2) and carbon dioxide (VCO_2) production measured using a paramagnetic oxygen analyser (model 570A, Servomex, UK), an infrared carbon dioxide analyser (model 1400, Servomex, UK) and a dry gas analyser (Harvard Apparatus, UK). Heart rate was measured by short-range telemetry (Polar F1, Finland). The speed and gradient needed to elicit 50% peak VO_2 for subsequent visits was determined on an individual basis from a nomogram predicting treadmill walking oxygen consumption (Workman et al, 1963).

At visit 2 and 3 a baseline blood sample was obtained. Resting heart rate was recorded and 5 min expired gases were collected. Subjects were then asked to walk on a treadmill at an intensity of 50% peak VO_2 for 90 min. At 30 min intervals heart rate was recorded, 2 min expired air samples were collected and a blood sample taken. At one visit subjects performed no resistance exercise before walking, and at the other visit the walking test was preceded by 20 min of high intensity resistance exercise. These second and third visits were carried out in random order. An additional blood sample was taken immediately on completing the resistance exercise.

Resistance exercise

Resistance exercise consisted of 2 sets of squats (each set with 20 repetitions followed by 30 pulses), 3 sets of press-ups (each set with 15 repetitions with the last repetition held for 20 seconds with the subjects face 1 inch off the floor), and 2 sets of

lunges on each leg (each set with 20 repetitions, followed by 30 lunge dips). This circuit was repeated until 20 minutes had elapsed.

Rate of fat and carbohydrate oxidation

Rates of fat and carbohydrate oxidation were calculated using the stoichiometric equations of Frayn as described previously in detail in chapter 4 (Frayn, 1983).

Blood collection and analysis

Blood samples were collected in cooled potassium EDTA tubes, placed on ice, and then centrifuged at 3000 rpm for 15 minutes at 4°C. The plasma was aliquoted and stored at –80 °C until analysis. Adiponectin was measured using a commercially available enzyme-linked immunosorbent assay kits (Quantikines, R&D systems).

Statistical analysis

Data are expressed as mean (± SEM). A repeated one-way analysis of variance (ANOVA) was used to compare differences between the measurements made at each time point during testing. Post-hoc analysis was performed with Student paired *t*-tests. Statistical significance was taken at the 5% level.

Results

Subject characteristics

The subjects body morphology and peak oxygen consumption are detailed in table 6.1.

Mean height (m)	1.81 ± 0.06
Mean weight (kg)	89.3 ± 8.3
Mean BMI ($kg.m^{-2}$)	27.2 ± 2.1
Percentage body fat (%)	17.0 ± 1.8
Mean fat free mass (kg)	74.0 ± 5.6
Mean fat mass (kg)	15.3 ± 3.0
Mean peak VO_2 ($l.min^{-1}$)	5.11 ± 0.46

Table 6.1 Body morphology and peak oxygen consumption.

Expired gas analysis and substrate metabolism

All subjects completed both 90-minute walking tests at 50% peak VO_2 (table 6.2) (figure 6.1 and 6.2). There was no significant difference in VO_2 at any time point between the walking test alone and the walking test preceded by resistance exercise. The rate of fat oxidation increased progressively during the 90-minute walking test ($p < 0.01$ for fat oxidation at rest vs 30 min, 30 min vs 60 min, and 60 min vs 90 min). Performing 20 minutes of resistance exercise prior to the 90 minute walking test significantly increased the rate of fat oxidation. The resting rate of fat oxidation was significantly higher after resistance exercise and remained higher than with walking alone at each time point during the test ($p < 0.01$ for resistance and walking vs walking alone at rest, 30, 60 and 90 min).

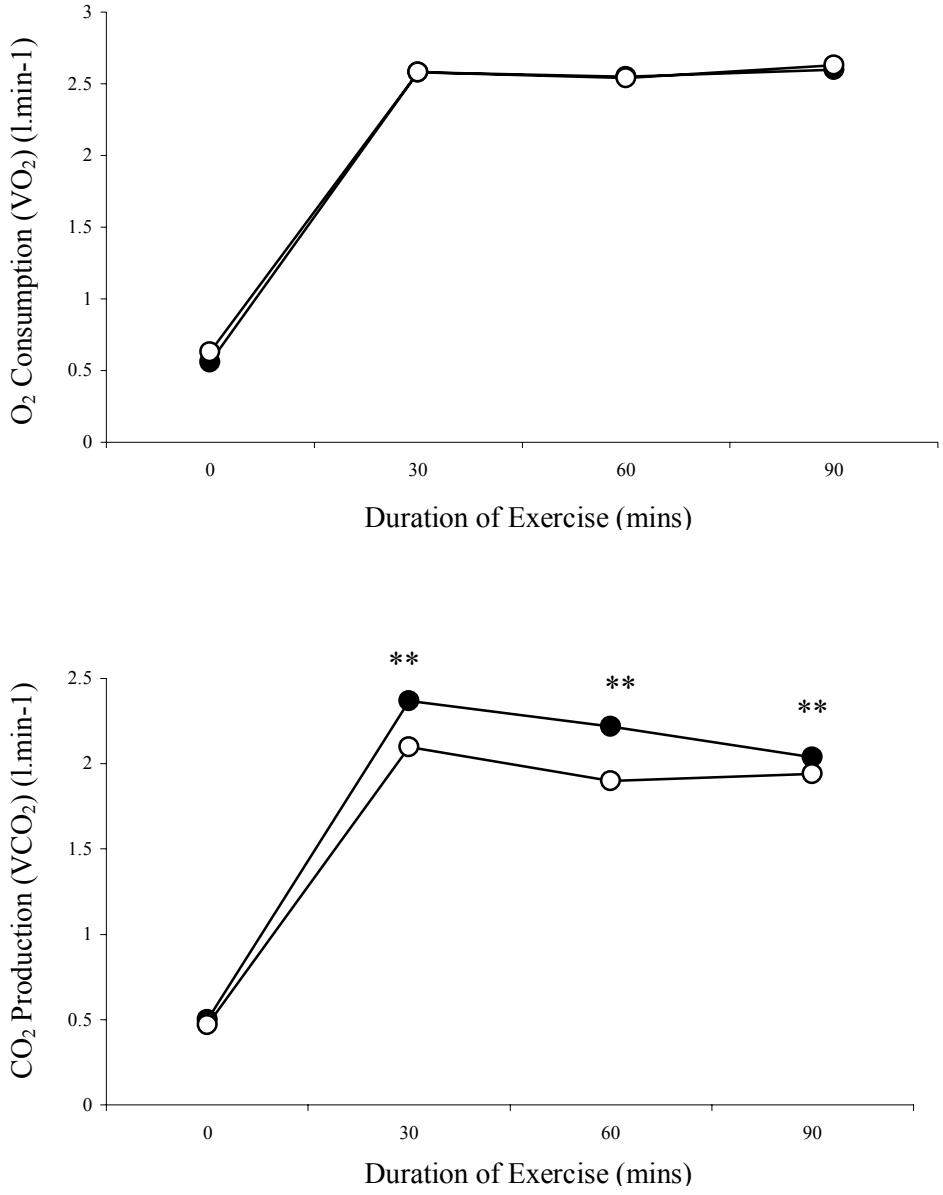


Figure 6.1. Expired gas analysis at rest and during exercise. ● Walking trial alone. ○ Walking trial after resistance exercise. Differences between groups assessed by ANOVA. Post-hoc analysis with student's paired t-test. **$p<0.01$ for walking compared to resistance + walking.

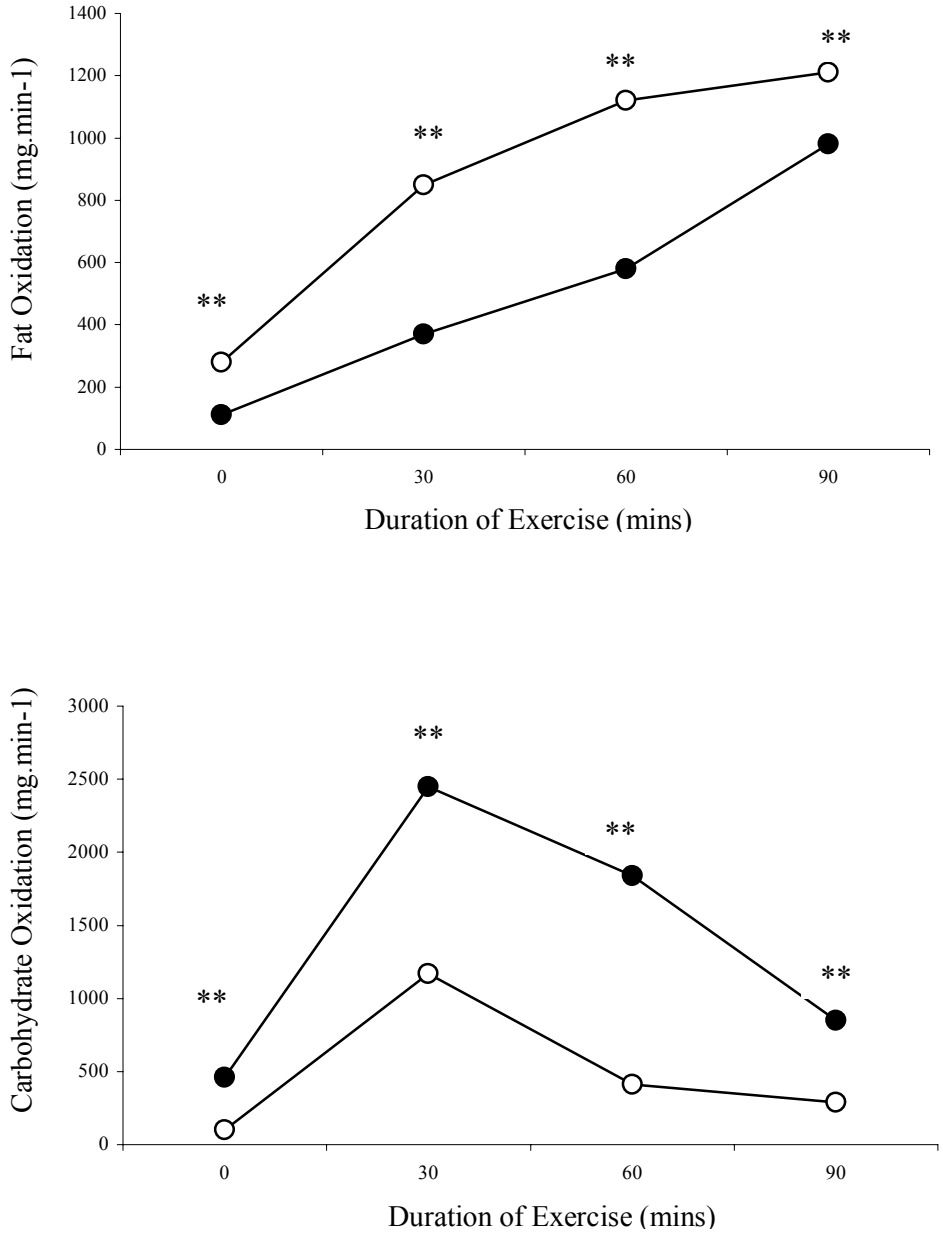


Figure 6.2. Rate of fat and carbohydrate oxidation at rest and during exercise.

● Walking trial alone. ○ Walking trial after resistance exercise. Differences between groups assessed by ANOVA. Post-hoc analysis with student's paired t-test. **p<0.01 for walking compared to resistance + walking.

	Walking	Resistance + walking	P value
Resting VO_2 ($l.min^{-1}$)	0.56 ± 0.18	0.63 ± 0.17	p>0.05
Resting VCO_2 ($l.min^{-1}$)	0.50 ± 0.14	0.47 ± 0.12	p<0.01
Resting Fat Oxidation ($g.min^{-1}$)	0.11 ± 0.02	0.28 ± 0.02	p<0.01
Resting CHO Oxidation ($g.min^{-1}$)	0.46 ± 0.03	0.10 ± 0.05	p<0.01
30 min VO_2 ($l.min^{-1}$)	2.58 ± 0.74	2.58 ± 0.61	p>0.05
30 min VCO_2 ($l.min^{-1}$)	2.37 ± 0.64	2.10 ± 0.37	p<0.01
30 min Fat Oxidation ($g.min^{-1}$)	0.37 ± 0.06	0.85 ± 0.07	p<0.01
30 min CHO Oxidation ($g.min^{-1}$)	2.45 ± 0.15	1.17 ± 0.13	p<0.01
60 min VO_2 ($l.min^{-1}$)	2.55 ± 0.68	2.54 ± 0.56	p>0.05
60 min VCO_2 ($l.min^{-1}$)	2.22 ± 0.75	1.90 ± 0.35	p<0.01
60 min Fat Oxidation ($g.min^{-1}$)	0.58 ± 0.06	1.12 ± 0.05	p<0.01
60 min CHO Oxidation ($g.min^{-1}$)	1.84 ± 0.18	0.41 ± 0.08	p<0.01
90 min VO_2 ($l.min^{-1}$)	2.60 ± 0.50	2.63 ± 0.52	p>0.05
90 min VCO_2 ($l.min^{-1}$)	2.04 ± 0.54	1.94 ± 0.32	p<0.01
90 min Fat Oxidation ($g.min^{-1}$)	0.98 ± 0.09	1.21 ± 0.09	P<0.01
90 min CHO Oxidation ($g.min^{-1}$)	0.85 ± 0.21	0.29 ± 0.18	P<0.01

Table 6.2. Expired gas analysis and rate of fat and carbohydrate oxidation.

Differences between groups assessed by ANOVA. Post-hoc analysis with student's paired t-test.

Plasma adiponectin response to exercise

Plasma adiponectin concentration increased during the walking test (figure 6.3). The difference became significant after 90 minutes of walking (Resting adiponectin 6.4 ± 0.7 μg/ml vs 90 min adiponectin 7.9 ± 0.4 μg/ml, $p < 0.01$).

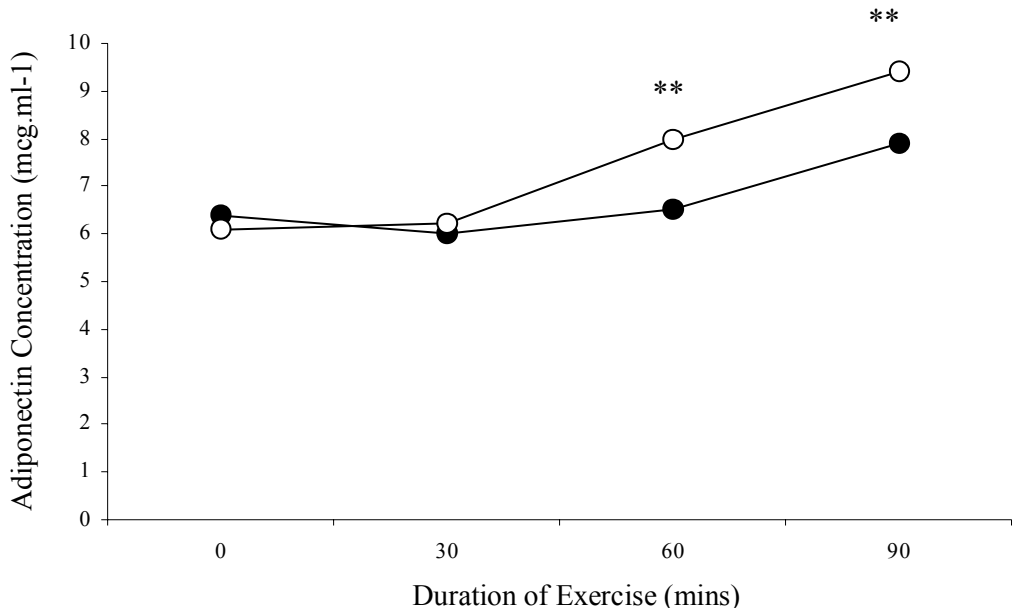


Figure 6.3. Increase in plasma adiponectin concentration during exercise. • Walking trial alone. ○ Walking trial after resistance exercise. Differences between groups assessed by ANOVA. Post-hoc analysis with student's paired t-test. **p<0.01 for walking compared to resistance + walking.

Plasma adiponectin concentration increased earlier and to a higher level during the walking test preceded by resistance exercise. There was no significant difference in adiponectin concentrations immediately after resistance exercise or after 30 min of walking, but at 60 min the difference became significant (Resting adiponectin 6.1 ±

0.5 μg/ml vs 60 min adiponectin 8.0 ± 0.4 μg/ml, $p < 0.01$). The plasma adiponectin concentration at 60 and 90 min after resistance exercise were significantly higher than those at 60 and 90 min after walking alone (8.0 ± 0.4 μg/ml vs 6.5 ± 0.5 μg/ml at 60 min; 9.4 ± 0.5 μg/ml vs 7.9 ± 0.4 μg/ml at 90 min, $p < 0.01$ for both comparisons).

Relationship between adiponectin concentration and rate of fat oxidation

Although both fat oxidation and adiponectin increased with walking, particularly after resistance exercise, no relationship was observed between the rate of fat oxidation and plasma adiponectin concentration ($r = 0.15$, $p > 0.05$ for walking alone; $r = 0.11$, $p > 0.05$ for walking and resistance exercise) (figure 6.4).

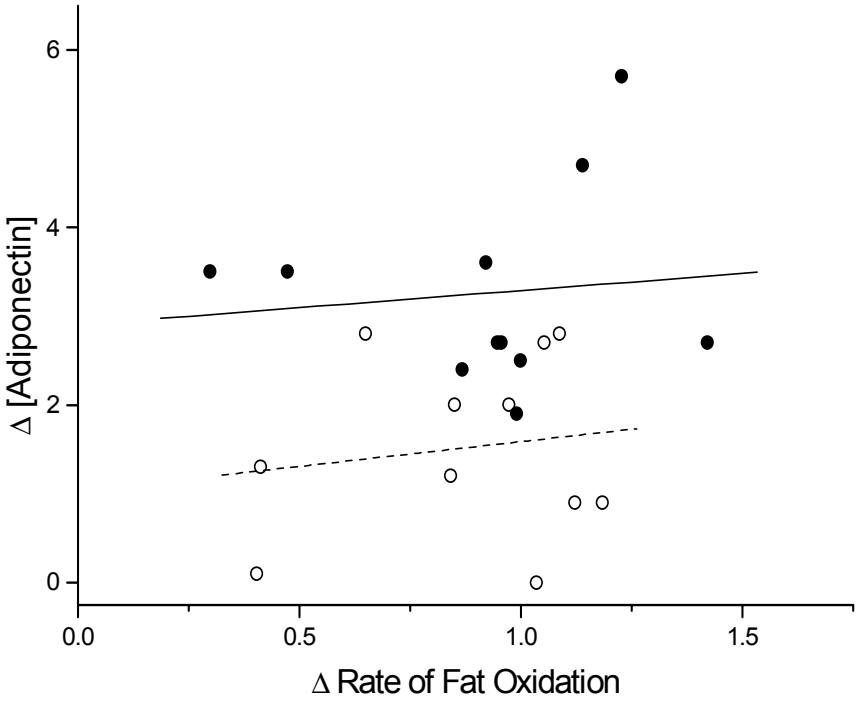


Figure 6.4. Relationship between plasma adiponectin concentration and rate of fat oxidation. Adiponectin concentration (mcg.ml-1). Rate of fat oxidation (g.min-1). ● Walking trial alone. ○ Walking trial after resistance exercise. Relationship analysed using linear regression.

Discussion

We found that our specifically designed exercise protocol of resistance exercise followed by prolonged, low intensity, aerobic exercise was successful in significantly increasing rates of fat metabolism. Plasma adiponectin increased during exercise, and more so during the protocol designed to maximise fat oxidation. Despite inducing both increased rates of fat oxidation and adiponectin concentrations, we found no association between them in these young healthy male subjects, in contrast to our observations in our cachectic heart failure patients.

Our finding that plasma adiponectin concentration increased with exercise is contrary to most of the previous studies, which have reported little or no change (Hulver et al, 2002; Yatagai et al, 2003; Ferguson et al, 2004; Jurimae et al, 2005; Klimcakova et al, 2006; Bobbert et al, 2007). Importantly, most of these studies used relatively short-term, high intensity exercise, which does not promote high levels of fat metabolism. Increased adiponectin has been previously demonstrated with prolonged strength training in diabetics and prolonged low to moderate intensity aerobic exercise in adults with a predisposition to the metabolic syndrome (Brook et al, 2006; Ring-Dimitriou et al, 2006).

These observations suggest that increased plasma adiponectin concentration during exercise does not directly promote fat oxidation, and that adiponectin secretion is not directly related to the rate of fat oxidation in healthy subjects. It appears that the sympathetic nervous system and catecholamines are key mediators of lipolytic activity during exercise (McMurray *et al*, 2005). Prolonged, low intensity exercise

has been shown to increase circulating catecholamines, induce lipid mobilisation, and increase the rate of fat oxidation with time (Moro *et al* 2004). Rate of fat metabolism during exercise has also been observed to relate to the degree of growth hormone release (Pritzlaff *et al*, 2000). While plasma BNP is known to rise following endurance exercise, it has previously been reported to show no significant response to conventional exercise testing in healthy controls (Ohba *et al*, 2001; Nicholson *et al*, 1993; Steele *et al*, 1997). In contrast, in patients with heart failure BNP appears to increase, from higher resting levels, during exercise testing (Maeder *et al*, 2007).

It would have been interesting to have measured plasma BNP response to this specific exercise protocol, and to have looked at how this related to rates of fat oxidation and adiponectin concentration. Furthermore, a similar study conducted in our main study population would have been of interest, although with the degree of exercise limitation in the cachectic heart failure patients prolonged exercise would not have been possible.

In conclusion, despite both rate of fat oxidation and plasma adiponectin concentration increasing significantly during exercise, we found no relationship between them. This suggests that adiponectin does not directly mediate fat oxidation, and its secretion is not directly related to the rate of fat oxidation in young healthy subjects during exercise.

CHAPTER 7

GENERAL DISCUSSION

The prognostic importance of developing cachexia in heart failure was first highlighted ten years ago by Anker *et al* (Anker *et al*, 1997). More recent analyses from the large SOLVD (Studies of Left Ventricular Dysfunction) and CHARM (Candesartan in Heart failure Assessment of Reduction in Mortality and morbidity) clinical trials have confirmed the strong relationship between weight loss or underweight status and impaired survival in heart failure (Anker *et al*, 2003; Kenchaiah *et al,* 2006; Kenchaiah *et al,* 2007).

Although epidemiological data remain limited, cachexia appears to develop in a significant proportion of heart failure patients, from 8.5% at 6 months in the CHARM program to 42% at 35 months in the SOLVD trial (both using a definition of cachexia as ≥5% weight loss) (Kenchaiah *et al,* 2006; Anker *et al*, 2003). With the increasing prevalence of heart failure and improved longer-term survival with contemporary drug and device therapy, cachexia is anticipated to become an even more significant problem in the future.

Muscle wasting has previously been assumed to be the major contributor to weight loss in heart failure, and has almost been considered to be synonymous with cachexia. The arbitrary, and easily clinically applied, definition of cachexia as a percentage loss of body weight over time has been widely employed in recent heart failure studies (Anker *et al*, 1997). The possible limitations of this definition and the importance of more detailed information regarding body composition was previously raised by a retrospective study by Lavie *et al,* who found that heart failure patients with clinical events, while having non-significantly lower BMI, had significantly lower percentage

body fat (Lavie *et al,* 2003). Furthermore, they found no difference in lean body mass on comparing event free survivors and those with clinical events, and a higher percentage body fat was the strongest independent predictor of event free survival. Interestingly, survival studies in chronic obstructive pulmonary disease (COPD) have shown a similar relationship between body mass and survival, with significantly higher mortality rates in underweight and normal-weight patients than in overweight and obese patients. More recent studies have shown that in COPD, in contrast to heart failure, fat-free mass is an independent predictor of mortality while fat mass is not (Schols AM et al, 2005). Therefore, contrary to previous suggestions, the nature of cachexia and how it develops appears to differ in these two chronic diseases.

In our cachectic heart failure patients we found a significant reduction in fat mass but no significant loss of fat free mass. Some previous studies of cardiac cachexia have found loss of both fat mass and fat free mass. It is possible that the development of cachexia in heart failure could initially involve the loss of fat, followed by muscle and bone, and that our study population were in the early stages of this wasting process. Alternatively, the fact that previous studies have involved heterogeneous populations of heart failure patients while our study specifically involved only patients with coronary artery disease (CAD) raises the question of whether the development of cachexia and balance of tissue loss may differ in patients with heart failure of different aetiologies.

In addition to finding no evidence of muscle wasting in our patients with cardiac cachexia, and despite increased concentrations of circulating cytokines, we found no

local activation of inflammatory cytokines or cell cycle regulators in skeletal muscle tissue. While our finding of increased circulating cytokines is consistent with previous studies, our muscle findings are contrary to the only other study quantifying cytokine expression in skeletal muscle in patients with heart failure (Levine *et al* 1990, McMurray 1991, Anker *et al* 1999, Gielen *et al* 2003). Importantly, Geilin *et al*, who found increased cytokine expression in muscle, studied a population of patients with predominantly idiopathic dilated cardiomyopathy (IDCM). Therefore a possible explanation for the discrepancy in our findings would be that, in contrast to patients with ischaemic heart disease, patients with IDCM have tissue as well as circulating cytokine activation.

Circulating levels of TNF-α are known to be increased in COPD, and even more markedly in the presence of cachexia (Takabatake *et al*, 2000; DiFrancia *et al*, 1994). Indeed, circulating TNF-α concentrations in cachectic COPD patients appear markedly higher than in our cachectic heart failure patients (COPD-cachexia 70.2 (100) pg/ml and HF-cachexia 27.2 (21.8-132.8) pg/ml) (DiFrancia *et al*, 1994). While this is an interesting observation we recognise the limitations of such a comparison. In the context of the conflicting findings of our study and Geilen *et* al, of even greater interest is a recent study measuring cytokines in the skeletal muscle of patients with 'weight stable' severe COPD using a human antibody array (Barreiro *et al*, 2007). Barreiro *et al* found significantly lower levels of TNF-α, and no difference in any of the other cytokines, in skeletal muscle of COPD patients compared to healthy controls, despite evidence of muscle weakness and oxidative stress.

Our finding of no muscle activation of cell cycle inhibitors is contrary to a previous study by our group reporting increased skeletal muscle expression of the cyclin dependent kinase (CDK) inhibitor p21 in COPD patients with cachexia. Importantly, the definition of cachexia widely used in COPD is more specific than in heart failure, requiring a reduction in fat-free mass in addition to body mass index (American Thoracic Society/European Respiratory Society, 1999). Thus, having found no significant loss of muscle mass in our cachectic heart failure patients the absence of activation of cell cycle inhibitors in the muscle is not surprising.

In addition to finding that the weight loss in our cachectic heart failure patients was predominantly due to loss of fat mass, we also found that they had altered energy metabolism, with higher rates of fat metabolism compared to heart failure patients without cachexia. This raised the question of what might be mediating this increased fat metabolism? While the literature supports a significant contribution of the sympathetic nervous system and catecholamines, it is clear that there are other important mediators involved: Both increased circulating TNF-α and adipose tissue derived TNF-α have been suggested to mediate depletion of adipose tissue mass in cachexia (Levine *et al*, 1990; McMurray *et al*, 1991; Dutka *et al,* 1993; Anker *et al,* 1997; Argiles *et al*, 1997; Kern *et al*, 1995; Warne *et al*, 2003); the adipose tissue specific hormone adiponectin is known to have an inverse relationship to body fat and induces weight loss in experimental animal models (Maeda *et al,* 1996; Chandran *et al,* 2003; Meier *et al,* 2004; Yamauchi *et al,* 2002; Bruun *et al,* 2003; Wang *et al,* 2006; Degawa-Yamauchi *et al,* 2005; Carey *et al,* 2006; Fruebis *et al,* 2001; Masaki *et al,* 2003); and the natriuretic peptides, well established as predictors of outcome

and markers of heart failure severity, appear to have additional lipolytic and lipid-mobilising actions (Sengenes *et al,* 2000).

We found that our cachectic heart failure patients, in addition to reduced body fat and increased fat metabolism, had markedly elevated serum adiponectin concentrations, and non-significantly elevated plasma BNP levels, compared to heart failure patients without cachexia. There was a positive association between adiponectin and BNP. Both negatively correlated with BMI and percentage body fat, and positively correlated with serum TNF-α concentration. There was a positive association between rate of fat oxidation and adiponectin concentration, but no relationship with BNP.

At the very least, our findings suggest preservation of the supposed physiological, inverse, relationship between adiponectin and fat mass in cachexia, and illustrate the role of BNP as a marker of disease severity (Chandran *et al,* 2003; Meier *et al,* 2004). While the relationship with TNF-α could be explained by the proposed anti-inflammatory role of adiponectin, TNF-α has been suggested to increase adiponectin secretion (Bruun *et al,* 2003; Wang *et al,* 2006; Degawa-Yamauchi *et al,* 2005; Carey *et al,* 2006). More interestingly, our findings raise the possibility that adiponectin, possibly under the influence of TNF-α and BNP, contributes to increased fat metabolism, loss of fat mass and the development of cachexia in heart failure. Alternatively, BNP could be the key mediator of increased lipolysis and loss of body fat, and in doing so promotes adiponectin secretion. A direct way of establishing the "cause and effect" relationship between increased adiponectin and BNP and reduced

body weight would be to examine change in weight, body fat and rate of fat metabolism following the administration of each peptide.

To determine whether there might be a causal relationship beween adiponectin and rate of fat metabolism we conducted an additional study in young healthy male subjects. This exercise study, designed specifically to maximise fat metabolism, found no direct relationship between adiponectin and the rate of fat oxidation. From this we concluded that adiponectin does not directly promote fat oxidation, and its secretion is not directly related to the rate of fat oxidation, in young healthy subjects during exercise. While this might suggest that the relationship we observed in our heart failure patients could be mediated by another factor, it is important to distinguish between a rapid, short-term change in substrate utilisation during exercise in healthy subjects and the chronic abnormal energy metabolism of heart failure.

There are a number of limitations in the investigations that contribute to this thesis. Due to the technical requirements of the project, and the not inconsiderable demands on the volunteers, the subject numbers are relatively small. In addition, patients with heart failure and cachexia are often so sick as to preclude inclusion in a study like this, introducing a selective bias towards patients with less severe disease. Subjects did not undergo a familiarisation treadmill test, which is known to produce more reliable exercise data. The IDCM subject group were recruited as an amendment to the original protocol and consequently had limited characterisation. It would have been interesting in light the conflicting findings with Geilen *et al* to perform skeletal muscle analysis in our IDCM group. Furthermore, it would have been interesting to

look at cytokine activation in the adipose tissue, but at the time the study was designed muscle wasting was considered to be the major manifestation of cardiac cachexia. Our assessment of energy metabolism would have been enhanced if we had also measured free fatty acids and urinary nitrogen. There were a number of methodological limitations discussed previously in the general methods section and results chapters. Finally, it is possible that the lack of significant difference observed between some of the biochemical parameters we measured might be the result of a type II error.

This work has raised a number of questions requiring further study. Are cachectic heart failure patients just those patients with the most advanced heart failure? Our cachectic population had all the markers of advanced heart failure with the lowest LVEF and peak VO_2, and the worst NYHA classification, but we know that there are patients with advanced heart failure who do not have cachexia. It would be interesting to compare heart failure patients matched for the established markers of disease severity with and without cachexia. Do cachectic heart failure patients initially lose body fat with subsequent muscle wasting? Is the change in body composition the same in patients with heart failure of different aetiologies? Both these questions could be addressed by prospectively monitoring changes in body composition in a population of heart failure patients. Are adiponectin and BNP mediators of the loss of adipose tissue? As suggested earlier this could be determined by a study examining change in weight, body fat and rate of fat metabolism following the administration of each peptide.

In conclusion, cachexia in patients with heart failure and stable coronary artery disease predominantly involves the loss of body fat, with no evidence of muscle wasting or inflammation. This is associated with elevated circulating levels of TNF-α, adiponectin and BNP, with the observed relationships between these peptides, and of each with body composition, suggesting that while this may be bystander phenomenology each may play an integral role in the altered energy metabolism, loss of body fat, and development of cachexia in heart failure.

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