THE UNIVERSITY *of* EDINBURGH

Molecular biomarker discovery and physiological assessment of skeletal muscle in cancer cachexia

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Doctorate of Philosophy (PhD)
The University of Edinburgh

2013

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Declaration

I declare that the work described in this thesis is entirely my own. Any contribution made by others to the work is acknowledged in the text. This thesis has not been submitted for any other degree or professional qualification.

Professor Kenneth CH Fearon; Clinical and Surgical Sciences (Surgery), University of Edinburgh principally supervised the thesis. Dr Carolyn A Greig; School of Clinical Sciences and Community Health, University of Edinburgh, and Professor James A Ross; Tissue Injury and Repair Group, University of Edinburgh provided additional supervision.

Nathan A. Stephens

Acknowledgements

I am both surprised and relieved to have reached the point of completing the final version of this thesis and I suspect many others share these emotions! Like climbing a mountain, there always seemed to be one more peak to go. The amount of work that unravelled as I progressed through my research was only successful thanks to the efforts and help of a huge number of people. I am indebted to all those who have assisted with the research work and/or helped with my sanity throughout the process.

First and foremost, I wish to thank Professor Kenneth Fearon for his supervision and mentorship; without which nothing would have been achieved. He has guided and imparted wisdom not just in academia, but also in the political minefield of surgery, art, wine, music and even an introduction to Renaissance history and the architecture and culinary delights of Tuscany. Dr Carolyn Greig was also a tremendous supervisor and fundamental in the success of the clinical studies along with helping me to 'keep my head while all about were losing theirs!'[1] I wish to thank Professor James Ross for support and guidance in his lab. His weekly research meetings were productive both in keeping up with progress and (more importantly) in educating me in the plethora of different biscuits and cakes available!

Other members of the Tissue Injury and Repair Group: Kathryn Sangster, Jim Black and Ian Ansell were also a real pleasure to have worked with and I appreciate their assistance, organisation and hard work. Thanks to the funding bodies – Cancer Research UK (CRUK), Royal College of Surgeons of Edinburgh, Union for International Cancer Control (UICC), Wyeth/Translational Medicine Research Collaboration for providing finance to allow all of the research work to be completed. I am grateful to all those who collaborated with me; particularly the efforts of Dr Iain Gallagher, along with Dr Denis Guttridge and his team, Professor James Timmons and Dr Calum Gray who all invested time in training me and imparting their research and laboratory skills. A special mention to radiographers Lynne Thomson and Claire Malone who went out of their way to help arrange MR scans often at very short notice. Thanks also to the UGI cancer specialist nurses and nursing staff in the Clinical Research Facility at the Royal Infirmary of Edinburgh (RIE) for help with patient recruitment and assessments. Progress of the clinical studies was greatly aided by support from the Consultant Surgeons and Registrars at RIE who helped with recruitment and biopsies. A particular vote of thanks to Mr Paterson-Brown, Mr De Beaux, Mr Tulloh, Mr Couper, Mr Ravindran, Ms Paisley, Mr Lamb, Mr Deans, Professor Garden, Professor Wigmore, Professor Parks, Mr Powell, Mr Hidalgo and Mr Duxbury. Thanks also to past and present members of the 'cachexia team' (Richard Skipworth, Benjamin Tan, Alisdair MacDonald, Neil Johns) for preparing the ground ahead of me, and for helpful advice and assistance.

[1] Paraphrased from 'If' by Rudyard Kipling, 1910

I would also like to give my sincere thanks to all those patients who gave up their time to take part in the research studies. The willingness and desire that they had to help contribute to scientific knowledge without the guarantee of direct personal benefit was striking. Their altruism and generosity were a real example of human goodness that is very much admired.

During my time in the lab, I was fortunate to work alongside a host of upstanding (and occasionally seated) researchers and fellow surgical trainees in addition to those mentioned above: John Terrace, Janet Kung, Grant Stewart, Joanna Brzeszczynska, Holger Husi, Bob Drummond, Jyoti Nanda, Jeffrey Lu, Linda Norrie, Raniska Tente and Joe Mee, amongst others who transiently joined the department. Coffee breaks in the lab were an essential part of maintaining sanity - the friendly banter, jokes and detours into the *avant-garde* were always enjoyable.

A big high-five to my kids, Tamara and Jakob, for encouragement, glasses of water, and reminding me about my real priorities in life. A special thanks to my parents and wider family for continuing love and support.

Above all, I wish to thank my beautiful wife, Hala. Her sacrifice of giving up chocolate until I completed my thesis was undoubtedly a more challenging task than my research and this thesis is dedicated to her sacrificial endurance. Her love, support and patience are undeserved yet thoroughly appreciated.

Abstract

Cachexia affects up to two thirds of all cancer patients with progressive disease. It is a syndrome characterised by weight-loss, anorexia, fatigue, asthenia, peripheral oedema, and is responsible for around 20% of cancer deaths. Cachectic patients suffer loss of both muscle mass and adipose tissue (with comparative sparing of visceral protein) and the lean tissue loss appears resistant to nutritional support. Progress in the treatment of cancer cachexia has been hampered due to poor understanding of the molecular mechanisms of skeletal muscle wasting in humans (rather than preclinical models) combined with a lack of accurate phenotyping particularly with respect to loss of skeletal muscle mass and function. The aim of the present thesis was to improve the knowledge and tools available for early intervention studies. The thesis focused on skeletal muscle as a key compartment in cancer cachexia. The experimental model was patients with upper gastrointestinal (UGI) cancer undergoing potentially curative surgery due to the associated higher incidence of cachexia along with the ability to access tissue biopsies. The thesis broadly divides into two sections. Part I reports a series of cancer cachexia biomarker discovery studies based on direct biopsy and analysis of human skeletal muscle. Part II focused on assessment and phenotyping of skeletal muscle mass and function in cachectic UGI cancer patients. In addition, the feasibility of longitudinal clinical studies that utilise such methodology is reported.

Intramuscular β-dystroglycan protein content (assessed using Western blot) was identified as a potential biomarker of cancer cachexia whereas changes in the structural elements of muscle (myosin heavy chain or dystrophin) appeared to be

survival biomarkers. Using transcriptomics, an 82-gene signature was demonstrated to correlate with weight-loss. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to examine the genes from this signature that were most upregulated. The exercise activated genes, CAMk2β and TIE1, correlated positively with weight-loss across different muscle groups (*Rectus abdominis, Vastus lateralis, Diaphragma*) indicating that cachexia was not simply due to inactivity and suggesting that these genes could be used as biomarkers of cachexia. None of the biomarkers discovered were consistent with pre-clinical models and therefore require further study before progressing to a validation programme. Electron microscopy of muscle biopsies demonstrated that the number and size of intramyocellular lipid droplets was increased in the presence of cancer and increases further with weight-loss/loss of adipose mass in other body compartments. The specific mechanisms and drivers of this phenomenon remain to be elucidated, but could relate to enhanced lipolysis or mitochondrial dysfunction in skeletal muscle as well as influencing muscle mechanical quality. Physiological assessment of patients with cancer cachexia established the negative impact that cachexia can have on muscle mass, function, muscle quality and quality of life, but demonstrated that the degree of impairment varies with sex and between muscle groups. Furthermore, the challenge of longitudinal studies in this patient group where frailty and clinical deterioration limit repeated assessments was highlighted. Such issues emphasise the need for a dual approach to the classification of cancer cachexia: if molecular markers prove difficult to discover or validate, then more specific and robust physiological indices of skeletal muscle mass and function may be the more important route to improve clinical trial design and cachexia classification.

Abbreviations

4E-BP, 4E binding protein
ActR, activin receptor
ADIPOQ, adiponectin, C1Q and collagen domain containing
AgRP, agouti-related peptide
AH, ascites hepatoma
Akt, Ak strain thymoma/protein kinase B
AMPK, adenosine monophosphate-activated protein kinase
ANGPTL7, angiopoietin-like 7
ANOVA, univariate Analysis of Variance
APC, adenomatosis polyposis coli
APCDD1, Adenomatosis Polyposis Coli Down-Regulated 1
Apo, apolipoprotein
APR, acute phase response
ATP, adenosine-5'-triphosphate
AUD, arbitrary units of densitometry
BC, Before Christ
BMI, body mass index
BNIP3, Bcl-2/adenovirus E1B 19kDa interacting protein 3
BP, blood pressure
BSA, bovine serum albumin
C, Celsius
CAMk, Ca^{2+}/calmodulin-dependent protein kinase
CART, cocaine and amphetamine regulated transcript
CASCO, cachexia score
cDNA, complementary deoxyribonucleic acid
CEA, carcinoembryonic antigen
cFT, calculated free testosterone
CoA, coenzyme A
COMP, cartilage oligomeric matrix protein
COPD, chronic obstructive pulmonary disease
CRP, C-reactive protein
CRUK, Cancer Research UK
CSA, cross-sectional area
CSF, cerebrospinal fluid
CT, computerised tomography
DEPC, diethylpyrocarbonate
DEXA, dual energy x-ray absorptiometry
DGC, dystrophin glycoprotein complex
DNA, deoxyribonucleic acid
DTT, dithiothreitol

E1, ubiquitin activating enzyme
E2, ubiquitin carrier protein
E3, ubiquitin protein ligase
ECL, enhanced chemiluminescence
EDTA, ethylenediaminetetraacetic acid
EE, energy expenditure
EGTA, ethylene glycol tetraacetic acid
eIF, eukaryotic initiation factor
ELISA, enzyme-linked immunosorbent assay
ENST, Ensemble transcript identifier
EORTC, European Organisation for Research and Treatment of Cancer
ERCP, endoscopic retrograde cholangiopancreatography
F, female
FA, fatigue
FAACT, Functional Assessment of Anorexia/Cachexia Therapy
FACIT, Functional Assessment of Chronic Illness Therapy
FACT-G, Functional Assessment of Cancer Therapy
FDA, Food and Drug Administration
FDR, false discovery rate
FFM, fat-free mass
FM, fat mass
FOXO, Forkhead box subgroup O transcription factor
FSH, follicle-stimulating hormone
FU, follow-up
GABRAPL1, gamma aminobutyric acid receptor-associated protein-like 1
GDF, growth and differentiation factor
GH, growth hormone
GLP, glucagon-like peptide
GO, Gene Ontology
GOBP, Gene Ontology biological processes
GPS, Glasgow Prognostic Score
Grb, growth factor receptor-bound protein
GSKβ, glycogen synthase kinase 3β
H&E, haematoxylin and eosin stain
Hb, haemoglobin
HC, healthy controls
HEPES, hydroxyethyl piperazineethanesulfonic acid
HGD, hand grip dynamometry
HGS, hepatocyte growth factor-regulated tyrosine kinase substrate
HINT3, histidine triad nucleotide binding protein 3
HIV, human immunodeficiency virus
HOMA-IR, homeostatic model assessment of insulin resistance

HSP90AB1, heat shock protein 90kDa alpha (cytosolic), class B member 1
HU, Hounsfield Unit
ICD, International Classification of Diseases
ICU, intensive care unit
ID, identification
IFN, interferon
IGF, insulin-like growth factor
IGFBP, insulin-like growth factor binding protein
IKES, isometric knee extensor strength
IKK, IκB kinase
IL, interleukin
IM, intermuscular
IPA, ingenuity pathway analysis
IRS, insulin receptor substrate
ISAK, International Society for the Advancement of Kinanthropometry
IκB, inhibitor of kappa-B
JAK, janus kinase
JNK, Jun N-terminal kinase
kDa, kiloDalton
KEGG, Kyoto Encyclopedia of Genes and Genomes
KPS, Karnofsky performance score
L3, 3rd lumbar vertebra
LBM, lean body mass
LC3, light chain 3
LD, lipid droplet
LH, luteinising hormone
LLEP, lower limb explosive power
LMF, lipid mobilising factor
LSB, Laemmli sample loading buffer
M, male
MAC-16, murine adenocarcinoma 16 mouse model
MAC, mid-arm circumference
MAFbx, muscle-specific F-box, also known as atrogin-1
MAMC, mid-arm muscle circumference
MAPK, mitogen-activated protein kinase
MAS, microarray suite software
MATLAB, matrix laboratory
MC4R, melanocortin 4 receptor
MCA, methylcholanthrene
MDT, multi-disciplinary team
Met-tRNA, methionyl transfer-RNA
MET, metabolic equivalent

MIC-1, macrophage inhibitory cytokine-1
MMP3, matrix metalloproteinase 3
MR, magnetic resonance
MRF, myogenic regulatory factors
MRI, magnetic resonance imaging
mRNA, messenger ribonucleic acid
mTOR, mammalian target of rapamycin
MURF-1, muscle-specific RING finger-1
MUST, Malnutrition Universal Screening Tool
MYBP-C, myosin binding protein-C
Myc, myelocytamotosis
MyHC, myosin heavy chain
MyLC, myosin light chain
MyoD, myoblast determination protein
N, Newton
N/A, not applicable
NF-κB, nuclear factor kappa-B
NHL, non-Hodgkin's lymphoma
NIH, National Institutes of Health
NLL, non-lymphocytic leukaemia
nNOS, neuronal nitric oxide synthase
NPY, neuropeptide Y
NSCLC, non-small cell lung cancer
NUDC, nuclear distribution C homolog
OGJ, oesophago-gastric junction
p70s6k, p70 ribosomal s6 kinase
PAGE, polyacrylamide gel electrophoresis
PBMC, peripheral blood mononuclear cell
PBS, phosphate buffered saline
PCK1, phosphoenolpyruvate carboxykinase 1
PDK1, phosphoinositide-dependent kinase-1
PET, Positron Emission Tomography
PF, physical function
PI3K, phosphatidylinositol 3-kinase
PIF, proteolysis-inducing factor
PIP3, phosphatidylinositol (3,4,5) trisphosphate
PKB, protein kinase B
PKR, double-stranded RNA-dependent protein kinase
PMSF, phenylmethanesulphonylfluoride
POLRMT, polymerase (RNA) mitochondrial (DNA directed)
POMC, pro-opiomelanocortin
PROX1, prospero homeobox 1

PSA, prostate specific antigen
Q.CSA, mid-femur quadriceps cross-sectional area
QLQ-C30, Quality of Life Questionnaire Core 30
QoL, quality of life
qRT-PCR, quantitative reverse transcription-polymerase chain reaction
RCAN1, regulator of calcineurin 1
RIE, Royal Infimary of Edinburgh
RMA, Robust Multi-array Average
RNA, ribonucleic acid
ROC, Receiver Operating Characteristic
ROI, region of interest
rpm, revolutions per minute
SAM, significance analysis of microarrays
SARM, selective androgen receptor modulator
SC, subcutaneous
SCLC, small cell lung cancer
SCRINIO, screening the nutrition risk of oncology patients
SD, standard deviation
SDS, sodium dodecyl sulphate
SEM, standard error of the mean
SF-36, Short Form-36 health survey
SGK1, serum/glucocorticoid regulated kinase 1
SHBG, sex hormone binding globulin
SI, systemic inflammation
SLC25A37, solute carrier family 25 (mitochondrial iron transporter), member 37
SNAP23, Synaptosomal-associated protein 23
SNARE, Soluble NSF Attachment Protein) REceptors
SOCS, Suppressor of Cytokine Signalling
SPSS, Statistical Package for Social Services
STAT, Signal Transducer and Activator of Transcription
STS, sit-to-stand time
TAM, tumour-associated macrophage
TBS, Tris-buffered saline
TBST, TBS Tween
TEM, transmission electron microscopy
TGF, transforming growth factor
TIE1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TNF, tumour necrosis factor
TNM, Tumour lymph Nodes and presence of Metastases staging system
TRAF, TNF receptor-associated factor
TSC2, tuberous sclerosis 2
TSF, triceps skinfold thickness

TT, total testosterone
TUG, 3m timed up-and-go
Ub, ubiquitin
UGI, upper gastrointestinal
UICC, Union Internationale Contre le Cancer
UPP, ubiquitin proteasome pathway
USB, Universal Serial Bus
VEGF, vascular endothelial growth factor
Vi, visceral
W, Watt
WBC, white blood cell
ZAG, zinc-α-2-glycoprotein

Chapter 1: **Introduction**

1.1 ***Overview***

Cachexia affects up to two thirds of all cancer patients (Laviano et al., 2005) and is also a feature of many other disease states including chronic obstructive pulmonary disease (COPD), chronic heart failure, chronic renal failure, rheumatoid arthritis and human immunodeficiency virus (HIV). It is a chronic wasting syndrome due to a combination of metabolic change, reduced food intake, reduced levels of physical activity, and increasing patient age (Skipworth et al., 2007). Weight-loss is one of the hallmarks of cachexia with depletion of both adipose tissue and lean body mass. In particular, there is a disproportionate loss of skeletal muscle. Patients may present with varying degrees of weight/tissue loss and it is important to consider cachexia as a journey rather than an event. A recent international consensus paper on cancer cachexia highlighted this continuum with a classification of three stages of cachexia: pre-cachexia, cachexia and the terminal state of refractory cachexia (Fearon et al., 2011). Cachexia is a significant cause of cancer patient morbidity and around 20% of cancer-related deaths are directly caused by malnutrition and cachexia due to immobility and cardio-respiratory failure (Tisdale, 2002). In spite of this, there are currently no evidence-based therapeutic agents in clinical use to reverse or even attenuate progression of cachexia in cancer patients. One of the major reasons for this is a limited understanding of the specific molecular mechanisms leading to the development of cancer cachexia combined with a scarcity of data describing the impact on muscle function and physical activity. A PubMed search (using search terms: ‘cachexia’ and ‘cancer’) indicates that over a quarter of publications relating

to cancer cachexia are review articles, highlighting the need for more primary investigations that shed light on the detailed mechanisms that produce the syndrome in patients. Furthermore, the majority of molecular hypotheses have been generated using pre-clinical models or reflect biochemical concepts and there has been little progress in relating these potential mechanisms to changes observed in the patient. With this background, the key theme of this thesis was to centre on skeletal muscle of patients with upper gastrointestinal (UGI) cancer, with or without cachexia, under two broad aims. Firstly, to stage cancer cachexia by investigating potential early skeletal muscle biomarkers relating to the presence or development of cachexia and; secondly, to assess the muscle phenotype of patients with cancer cachexia.

The introductory section of this thesis gives an overview of cancer cachexia and current understanding of the biological processes involved. The concept and potential of biomarkers in general will be described along with an outline of current data relating to biomarkers of cancer cachexia. This provides a background and rationale for the subsequent investigations described in the rest of the thesis.

*1.2 **Cancer cachexia***

1.2.1 **What is cachexia?**

The word cachexia is derived from the Greek ‘kakos’ meaning ‘bad’ and ‘hexis’ meaning ‘condition’, ‘habit’ or ‘state of being’. It has been recognised for many years as a complex syndrome with an associated poor outcome. Indeed, the Greek physician Hippocrates described cachexia in the third century Before Christ (BC):

> "the flesh is consumed and becomes water,…the abdomen fills with water; the feet and the legs swell, the shoulders, clavicles, chest and thighs melt away…The illness is fatal.".
>
> Hippocrates, 460-370 BC

Cachexia is a clinical syndrome characterised by anorexia, early satiety, severe weight-loss, weakness, anaemia and oedema. It is due to a combination of reduced energy intake and metabolic change. Cachexia can be recognised easily in its advanced stage, but is much more difficult to predict for the purposes of early intervention. In the past, cachexia was poorly defined and publications in the field used arbitrary degrees of weight-loss over differing time periods to categorise cachectic cohorts. This conundrum was highlighted in an article investigating the cachexia-anorexia syndrome in which the authors (Lasheen and Walsh, 2010) commented on the various definitions of cachexia used in several, mainly therapeutic, studies:

Weight-loss:

>10% of pre-illness weight (Feliu et al., 1992)

>10% in past 6 months (Fietkau et al., 1997)

>5% of pre-illness weight (Bruera et al., 1999)

>5% in previous 6 months (Strasser F, 2002)

>5% in previous 6 weeks (Rowland et al., 1996)

>5 pounds weight in last 2 months (Loprinzi et al., 1994)

Part of the difficulty for researchers has been that understanding of the basic characteristics and molecular mechanisms leading to cachexia and its subsequent impact on patients is limited. However, the lack of an agreed definition has hampered interpretation and comparison of individual studies, along with limiting identification and treatment of cachectic patients and the development of therapeutics.

Patients with 10% or more weight-loss from pre-illness weight are generally considered malnourished/wasted and this cut-off is used to classify patients as cachectic in many studies. Furthermore, 10% weight-loss is associated with higher surgical risk (Windsor and Hill, 1988) and has more substantial systemic effects on lung function, poorer laboratory nutritional measures and worsened survival in pancreatic cancer patients compared with using lower weight-loss values (Bachmann et al., 2009). However, in recent years, there has been a concerted effort to come up with more meaningful and relevant definitions based on current evidence. As knowledge about cancer cachexia evolves, these definitions will no doubt be refined, but at present they represent an effort to provide consistency to allow more meaningful collaborative research and interpretation of larger data sets. A summary of each of these definition papers follows:

Definition of cancer cachexia: effect of weight-loss, reduced food intake, and systemic inflammation on functional status and prognosis (Fearon et al., 2006). This was the first paper to attempt a considered definition of cancer cachexia related to the multidimensional nature of the syndrome rather than a crude weight-loss cut-off. It utilised a cohort of patients entered into a separate nutritional trial and

analysed a definition of weight-loss alone or incorporating additional measures of nutritional intake and inflammation. All patients had a weight-loss >5% and the study divided the patients according to weight-loss above or below 10%. Using weight-loss alone, >10% as a definition did not distinguish the groups according to subjective measures of quality of life (QoL), but they did have significantly different hand grip strength and Karnofsky performance scores (KPS). When patients were stratified by combining weight-loss >10%, reduced food intake <1500 kcal/day and systemic inflammation with a C-reactive protein (CRP) > 10mg/l then cachectic patients had significantly poorer subjective and objective measures of QoL, function, performance status and survival.

Cachexia: a new definition (Evans et al., 2008).

This paper described the consensus from a meeting of cachexia researchers in 2006. The definition was:

> "cachexia, is a complex metabolic syndrome associated with underlying illness and characterised by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight-loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with wasting disease. Wasting disease is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity."

In order to make this definition more meaningful for practical use, the diagnostic criteria were refined to:

> "weight-loss of at least 5% in 12 months or less in the presence of underlying illness, plus three of: decreased muscle strength, fatigue, anorexia, low fat-free mass index or abnormal biochemistry (increased inflammatory markers: CRP >5.0mg/l or 1L-6 >4.0 pg/ml, anaemia: Hb <12 g/dl, or low serum albumin: <3.2 g/dl)."

The paper also suggested that cachexia could be described as mild (weight-loss >5%), moderate (>10%) or severe (>15%) over the preceding 12 months. In cases where weight-loss was not able to be assessed, a body mass index (BMI) <20 kg/m^2 was thought to be adequate to represent cachexia. This definition was broadly related to cachexia of any aetiology (i.e. cancer, cardiac failure, COPD, HIV), was not evidence-based, and did not take into account potential differences between each of these disease aetiologies.

Consensus definition of sarcopenia, cachexia and pre-cachexia: joint document elaborated by Special Interest Groups "cachexia-anorexia in chronic wasting diseases" and "nutrition in geriatrics" (Muscaritoli et al., 2010).

This definition was based on answers of experts in the field to a series of questions. Cachexia was defined as:

> "a multifactorial syndrome characterised by severe body weight, fat and muscle loss and increased protein catabolism due to underlying disease/s'."

The authors suggested that, due to the wide range of clinical manifestations associated with cachexia, staging is warranted. The consensus agreed that inflammation has a role in the pathogenesis of cachexia, cachexia is different to malnutrition but anorexia can significantly contribute to the nutritional deterioration

of cachexia if it is not properly treated. The authors suggested utilising an adapted questionnaire for anorexia. There was also agreement that cachexia was associated with changes in body composition – most importantly loss of skeletal muscle. The importance of staging cachexia was emphasised and a proposal to stratify patients into pre-cachectic or cachectic was outlined. Pre-cachexia was defined as the presence of all the following factors: underlying chronic disease, unintentional weight-loss >5% over the preceding 6 months, chronic/recurrent systemic inflammatory response, anorexia or anorexia-related symptoms. The authors suggested that obese people can have pre-cachexia but it may be difficult to diagnose and commented on the concept of sarcopenic obesity.

Definition and classification of cancer cachexia: an international consensus (Fearon et al., 2011).

This paper describes a definition specific to cachexia caused by cancer. The definition was reached after focus group meetings and two Delphi rounds. It defined cancer cachexia as:

> "a multifactorial syndrome characterised by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. The pathophysiology is characterised by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism."

Diagnostic criteria for cancer cachexia were defined as:

> "weight-loss >5% over the preceding 6 months (in absence of simple starvation); or BMI <20 kg/m^2 and any degree of weight-loss >2%; or

appendicular skeletal muscle index consistent with sarcopenia (males <7.26 kg/m^2; females <5.45 kg/m^2) and any degree of weight-loss >2%."

The values for appendicular skeletal muscle are based on Computed Tomography (CT) cross-sectional imaging of skeletal muscle at the 3rd lumbar vertebra (L3) level. Alternative measures that the authors suggested were bioimpedance analysis, or mid-arm muscle area by anthropometry. Importantly, this paper also highlighted the need to consider the stages of cancer cachexia; pre-cachexia, cachexia and refractory cachexia. These are key points to consider as the treatment type and aggressiveness will be determined according to where the patient is on the cachexia journey.

Even though these definitions are in their infancy, there have already been groups publishing descriptions of potential scoring systems to categorise the severity of cachexia.

The cachexia score (CASCO): a new tool for staging cachectic cancer patients (Argiles et al., 2011).

This descriptive paper outlined proposals for a scoring system based on five domains: measures of weight-loss and body composition, inflammation/metabolic abnormalities and immunosuppression, physical performance, anorexia and QoL. However, the score has not been validated in patients and concrete evidence for a relationship between some of the proposed plasma markers and cachexia is lacking (Baracos, 2011).

Defining and classifying cancer cachexia: a proposal by the SCRINIO (Screening the nutrition risk of oncology patients) Working Group (Bozzetti and Mariani, 2009).

The authors of this paper chose a cut-off of 10% weight-loss to distinguish pre-cachexia and cachexia with the addition of the presence or absence of symptoms (anorexia, fatigue or early satiety). This resulted in a severity staging system with four classes of cachexia. Whilst the authors did examine the scoring system in 1307 of their cancer outpatients, it has not, to date, been validated in any other independent cohorts.

The motivation behind the development of cachexia scoring/staging systems is positive. However, cachexia definitions have only emerged recently and there is ongoing development of and discussion about them amongst researchers. It is therefore perhaps premature to propose scoring systems until the appropriate evidence base, measurement tools and statistical methodology is available, has been agreed upon and validated (Baracos, 2011).

1.2.2 **Epidemiology and demographics**

Weight-loss is seen as the hallmark of cachexia and commonly triggers patients to consult a physician. At diagnosis, one third of all cancer patients will have lost >5% of their original body weight and the overall incidence of weight-loss in the general cancer population is 63% (Laviano et al., 2005). Taken together with the fact that one in three individuals will develop cancer in their lifetime, these figures highlight

the importance of cachexia and the necessity to improve our understanding of the field.

1.2.2.1 **Impact of tumour type**

Although cachexia can occur in patients with cancer affecting any organ, there is a considerable degree of variation in the frequency of weight-loss associated with different tumour types. This is evident from the results of a study of cancer patients by the Eastern co-operative oncology group in the 1980s (Dewys et al., 1980). Table 1.1 shows the results.

Of particular note is the aggressive weight-loss associated with pancreatic cancer with the values in the table relating to weight-loss in the preceding 2 months (whereas it is over 6 months for the other tumour types).

A more recent study (Fox et al., 2009) investigated variations in the incidence of cancer cachexia according to four different definitions. The study included 8451 cancer patients (mixed tumour types) who were classified as cachectic according to (1) International Classification of Diseases (ICD) definition alone, (2) ICD codes with addition of anorexia, abnormal weight-loss and feeding difficulties, (3) prescription of certain 'anti-cachectic' drugs, or (4) post-cancer diagnosis weight-loss >5%. The incidence of cachexia was much lower than reported in the 1980s (Table 1.1) study (2.4%, 5.5%, 6.4%, 14.7% according to the different definitions respectively).

Table 1.1: Frequency of weight-loss according to tumour type

Tumour type		Weight-loss*			
		None	0-5 %	5-10 %	>10 %
Favourable NHL		69	14	8	10
Breast		64	22	8	6
Acute NLL		61	27	8	4
Sarcoma		60	21	11	7
Unfavourable NHL		52	20	13	15
Colon		46	26	14	14
Prostate		44	28	18	10
Lung	Small cell	43	23	20	14
	Non-small cell	39	25	21	15
Pancreas		17	29	28	26
Gastric	Nonmeasurable	17	21	32	30
	Measurable	13	20	29	38

*Adapted from Dewys et al (Dewys et al., 1980). *Data refers to % weight-loss over the preceding six months apart from pancreatic cancer, which is over the preceding two months. Numbers refer to percentage of total patient number for each tumour type. Abbreviations: NHL, Non-Hodgkin's lymphoma; NLL, non-lymphocytic leukaemia.*

The authors estimated that 23% of cancer patients might have cachexia at some point after their cancer diagnosis as determined by any of the four definitions. The tumour types that were most associated with cachexia by any of the definitions were: breast (26.5%), prostate (25.7%), lung (20.3%) and colorectal (11.7%). Of note, only 3.9% of pancreatic cancer patients were cachectic by any of the definitions. These percentages contradict previous reports (Table 1.1) and also the majority of recent publications where, for example, pancreatic cancer is associated with cachexia in ~80% of patients (Fearon et al., 1999). These conflicting results may be due to problems with data capture using ICD codes, which are dependent on a physician entering the details. There are many papers in a variety of diseases highlighting inaccuracies in the ICD classifications due to its subjective nature and what type of information is put in (Zeng and Bell, 2011, Hagen et al., 2009, Gibson and Bridgman, 1998, Campos-Outcalt, 1990, Lloyd and Rissing, 1985, Hsia, 1990), and it is likely that this has impacted to some degree on the results of the study. Furthermore, one of the four definitions (definition 4) relied on post-diagnosis weight-loss (in contrast to other studies which generally report pre-diagnosis weight-loss) and there was no comment on any different treatment modalities that may have influenced progression of cachexia (i.e. did they have chemotherapy or resectional surgery?). It is therefore likely that the methodology of the study has yielded misrepresentative results.

The percentage of weight losing individuals seems to be highest in patients diagnosed with pancreatic or gastric cancer (>80%) (Dewys et al., 1980). In one study, 85% of patients with newly diagnosed unresectable pancreatic cancer lost a median of 14% of their pre-illness stable weight, increasing to a median of 25% near

the time of death (Wigmore et al., 1997b). Indeed, the prevalence of weight-loss in cancer patients has been quoted as high as 86% in the last 1-2 weeks of life (Teunissen et al., 2007).

Why pancreatic or gastric cancer should be more strongly associated with cachexia than tumours such as breast cancer is still not clear (Tisdale, 2005). One possibility is that certain tumours are more likely to present earlier in the disease process prior to the development of weight-loss or any systemic symptoms. For example, weight-loss may be the first and only presenting symptom of a patient with pancreatic cancer whereas a breast tumour or lymphoma may be noticed by the patient as a lump during self-examination when the tumour is at an earlier stage and prior to changes in body composition. Alternatively, precipitation of an acute phase response with resultant hypermetabolism and loss of skeletal muscle proteins may be more common in certain tumour sites (e.g. pancreatic cancer (Fearon et al., 1999)).

It is also important to consider that along with the differences in prevalence of cachexia between different tumour sites, there is also variation within individual types of tumour. For example, although up to 85% of pancreatic cancer patients develop cachexia, 15% will not (Tisdale, 2009). The reasons for this remain unclear, but it raises the intriguing possibility of a cachexia resistant phenotype or genotype.

1.2.2.2 **Impact of gender**

It is well recognised that men have a different body composition to women and the prevalence of each tumour type varies between genders. For example, Cancer

Research United Kingdom (CRUK) data shows that in 2009 the top 5 cancers for men were: prostate, lung, bowel, bladder and NHL, whereas for women it was: breast, bowel, lung, uterus and ovary (Website, 2009). Likewise, sex-specific differences in survival for similar cancer types have been reported (Skipworth et al., 2011, Oberaigner and Siebert, 2011). It might therefore be supposed that the likelihood and impact of cancer cachexia would also vary according to gender. However, there are few studies that have investigated this directly and many others have not taken it into consideration in the interpretation of results, but rather have pooled data for men and women.

In an animal model of cardiac cachexia using Sprague Dawley rats, body weight was seen to decline in male rats following myocardial infarction surgery, but not in females (Palus et al., 2009). Similarly, in a mouse model of cancer cachexia (colon-26 tumour), males demonstrated a more severe phenotype. Male mice had greater and more rapid loss of overall body weight, skeletal muscle and cardiac muscle along with an increased mortality compared with female mice (Cosper and Leinwand, 2011). Furthermore, in the same paper, male cachectic mice had poorer survival and increased markers of autophagy compared with the females. The administration of an oestrogen antagonist to females led to a cachectic phenotype similar to the males suggesting a role of the gonadal hormones in this sexual dimorphism. Likewise, in a study of transgenic mice overexpressed with myostatin (thought to be an inhibitor of skeletal muscle mass) in skeletal muscle, male mice demonstrated lower muscle mass, reduced muscle fibre size and decreased numbers of myonuclei along with

decreased cardiac muscle mass and increased fat mass (Reisz-Porszasz et al., 2003). However the same changes were not apparent in female mice.

In humans, there is also evidence of sexual dimorphism in body composition changes with weight-loss and cachexia. In obese 'healthy' patients undergoing a weight-loss program, although both genders lost similar amounts of weight and body fat, men lost more visceral fat than women whereas subcutaneous fat was depleted more in women than men (Wirth and Steinmetz, 1998). In HIV patients, an autopsy study demonstrated that women had a lower frequency of cachexia than men, although died at a younger age (Morgello et al., 2002). Observations in lung cancer patients have shown that significant muscle wasting is twice as prevalent in males compared with females (Baracos et al., 2010). Indeed, weight-loss (including muscle loss) has been suggested to play a role in mediating sex-related differences in lung cancer patients' survival (Palomares et al., 1996, Chlebowski et al., 1996). Likewise, female gender has been shown to be a key determinant of weight stabilisation, with resultant improved QoL and survival duration, in a post hoc analysis of data from patients with unresectable pancreatic cancer enrolled in a multicentre nutritional intervention study (Davidson et al., 2004).

There may also be sex differences in the impact of cachexia on muscle function. In an immobilisation study, relative to the loss of muscle mass, females sustained a proportionally greater loss of strength than males (Yasuda et al., 2005). Similarly in a cohort of hospitalised patients with mixed disease aetiologies, handgrip strength

was demonstrated to decrease significantly more in men with severe weight-loss than in women (Norman et al., 2012).

1.2.2.3 **Impact of ageing**

It is generally accepted that increasing age is associated with increased frailty. This is due in part to the age related degenerative loss of skeletal muscle known as sarcopenia (Rosenberg, 1997). However, this loss of muscle may not be obvious with conventional clinical assessment tools. This was illustrated in a study of longitudinal body composition changes in healthy men age 20-96 years (Jackson et al., 2012). The authors showed that BMI, fat mass, and percentage of body fat increased between 20-80 years before reaching a plateau, whereas fat-free mass (including skeletal muscle) peaked at 47 years and then declined with ageing. However, although BMI (a conventional anthropometric assessment tool) reflected the increase in fat mass that occurred with healthy ageing, it was unable to identify the loss of fat-free mass that started at age 47 years (Jackson et al., 2012). This suggests a need for more sophisticated measures of body composition studies in sarcopenia or cachexia, rather than only including anthropometric assessments.

Given that the average age of cancer patients is 60-70 years, it is probable that age related loss of muscle would be relevant. Furthermore, the process of sarcopenia is exacerbated by chronic illness, inadequate diet and bedrest (Cuthbertson et al., 2005, Coker and Wolfe, 2012), and it is likely that the presence of systemic inflammation/cachexia will exacerbate this muscle loss.

1.2.2.4 **Impact of obesity**

There is an increasing level of obesity in the general population (especially in Western cultures) and obesity is itself a risk factor for the development of cancer. It is important to recognise that underneath the mantle of fat there may be clinically occult muscle wasting. This trend to what has been termed 'sarcopenic obesity' in advanced cancer patients has recently been emphasised (Prado et al., 2008). Indeed, in a study of patients with advanced pancreatic cancer, CT defined sarcopenic obesity was shown to be an independent determinant of reduced survival (Tan et al., 2009).

This has added a further layer of complexity in the clinical assessment of such obese individuals and there has been a paradigm shift towards the concept of the cachectic, but overweight, cancer patient. There has also been recognition that conventional anthropometric measures are probably inadequate in identifying at risk individuals and therefore utilising more detailed imaging modalities such as CT or Magnetic Resonance (MR) imaging may be necessary.

1.2.3 **Symptoms of cancer cachexia**

Whilst weight-loss and muscle loss may be predominant features of cachexia, patients present with a spectrum of symptoms. In a study examining the symptoms prevalent in cancer patients with cachexia-anorexia syndrome (defined as weight-loss >10% and anorexia of any severity), 9 out of the 26 symptoms assessed were found to be distinctly related to cachexia: early satiety, constipation, nausea, taste

changes, vomiting, dysphagia, fatigue, weak, and lack of energy (Lasheen and Walsh, 2010). Likewise, a recent systematic literature review looking at the association of involuntary weight-loss with a variety of domains related to cachexia found patients with weight-loss to have more depression, abdominal fullness, taste changes, vomiting, mouth dryness, dysphagia and loss of appetite (Blum et al., 2011). These symptoms appear quite non-specific and can be present in many other disease conditions. There will also be overlap, of course, between the symptoms related to the underlying cancer and those of cachexia. For example, anorexia may be a common symptom of patients with cachexia, but in those who have oesophageal or head and neck tumours, the effect will be exacerbated by dysphagia/odynophagia and anatomical obstruction to food related to the site of the tumour itself. Similarly patients with colorectal malignancies may have constipation or a feeling of abdominal fullness associated with impending obstruction of the colon by tumour bulk. Many of the symptoms related to cachexia are caused or exacerbated by the presence of the systemic inflammatory response and this will be discussed further in Section 1.2.6.2. Clinical history and examination remain the fundamental tools in diagnosing patients with cachexia and malnutrition. Advanced cachexia is easily recognised, but diagnosing cachexia in its early stages remains challenging. Although different symptoms predominate in individual patients and may also change with time, weight-loss, anorexia and fatigue/weakness are among the commonest symptoms reported and are often the triggers that spur patients to attend their physician. These three symptoms in the context of cachexia will be discussed in more detail in the next few paragraphs.

1.2.3.1 WEIGHT-LOSS

Weight-loss is seen as the hallmark of cachexia and may be the only change that triggers a patient to consult their physician. It is due to the combined loss of both lean and adipose tissue and it should be remembered that the magnitude of functional tissue loss may be masked by the presence of peripheral oedema/ascites or even increased visceral mass due to tumour (Lieffers et al., 2009). As previously discussed, the incidence of weight-loss in the general cancer population is 63% (Laviano et al., 2005) and this increases to over 80% for patients diagnosed with pancreatic or gastric cancer (Dewys et al., 1980). Weight-loss >5% suggests developing cachexia, while patients with >15% weight-loss are well advanced into the cachectic state. The presence of weight-loss has long been recognised as a poor prognostic factor in cancer patients. In patients with gastrointestinal cancer, severe wasting may account for 30-50% of deaths and up to 80% of deaths in patients with advanced pancreatic cancer (Davidson et al., 2004, Nelson, 2000, Palesty and Dudrick, 2003, Ripamonti, 1999). Furthermore, weight-loss may also be associated with poor prognosis in cancer patients who are staged initially as having curative disease. A recent study of 227 patients undergoing surgery for pancreatic cancer demonstrated that 40.5% of patients were cachectic pre-operatively (as defined by weight-loss >10%) (Bachmann et al., 2008). A total of 150 of these patients underwent tumour resection with curative intent and the remaining 77 underwent a palliative operation due to the presence of locally advanced disease or metastases. The successful tumour resection rate was significantly different between cachectic and non-cachectic patients (48.9% vs. 77.8% respectively, $p<0.001$). In those patients who underwent a resection with curative intent, mean survival was 451 days

in patients with pre-operative weight-loss compared to 654 days for those who had not lost weight (p=0.001).

The individual contributions of muscle versus adipose tissue depletion to the total weight-loss in cancer patients is unclear, but has recently gained interest. Many studies confirm loss in both body compartments (Lieffers et al., 2009, MacFie and Burkinshaw, 1987, Sarhill et al., 2003), but there have been others in human cancer cachexia demonstrating predominant fat loss (Fouladiun et al., 2005, Heymsfield and McManus, 1985) that has clouded understanding of the process. Part of the difficulty in interpreting and comparing such studies is that methodologies and cachexia definitions differ, and some of these results may be more reflective of simple starvation than of cachexia (Blum et al., 2011). However, data exist to suggest that there are differences in the magnitude and time course of alterations in muscle and fat compartments (Fearon, 2008, Abdiev et al., 2011, Kiyama et al., 2005, Fouladiun et al., 2005). Additionally, a recent study using a murine model of cancer cachexia reported that genetic ablation of lipases not only prevented increased lipolysis but, unexpectedly preserved skeletal muscle mass in the mice (Das et al., 2011). This indicates that the physiological interplay between fat and muscle in human cancer cachexia may be more complex and more significant than previously thought (Johns et al., 2012, Fearon, 2011). Future studies using consistent definitions and more sophisticated body composition analyses are likely to shed light on this issue.

1.2.3.2 ANOREXIA

Anorexia is the loss of the desire to eat associated with a reduced food intake. An

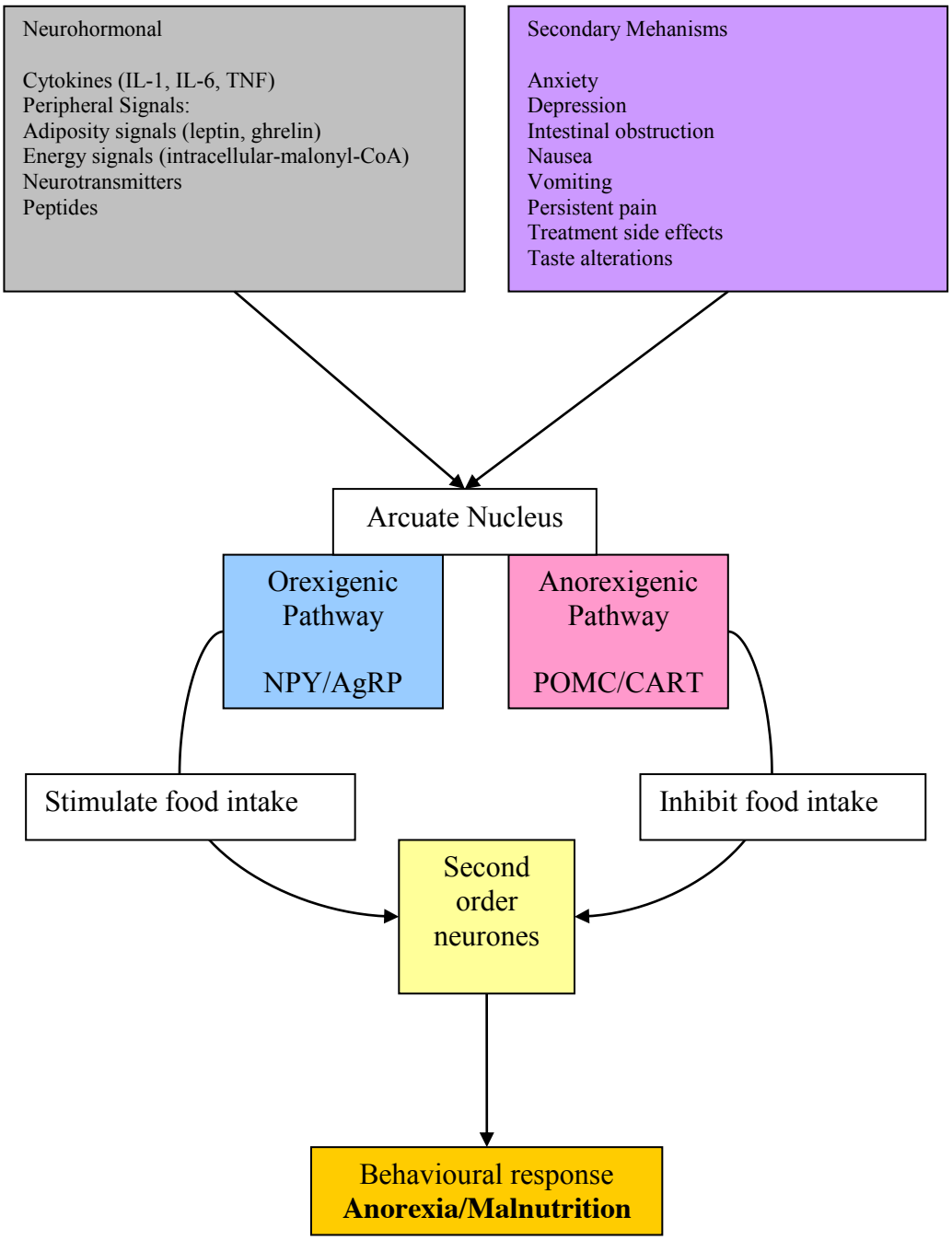
inadequate net nutritional intake occurs frequently in cancer patients who fail to increase their intake appropriately to cope with increasing resting energy demands. The relationship between anorexia and weight-loss is not clear-cut. Whilst many studies have reported a strong correlation between the two, in patients with weight-loss >10%, 39% of individuals did not have anorexia, 16% of patients had a normal food intake and 12% had anorexia without any weight-loss (Blum et al., 2011). The presence of anorexia with or without weight-loss is associated with poor QoL, low performance status and poorer survival (Chang et al., 2005) and is an independent risk factor for poor prognosis in lung cancer (Hoang et al., 2005), prostate cancer (Collette et al., 2004) and terminally ill cancer patients (Maltoni et al., 1995).

Body weight and BMI have been used as traditional indicators of nutritional status, but with the increasing tendency towards obesity in the West, and the possible presence of compounding factors such as oedema or ascites, these markers may be normal even in a patient who is cachectic and malnourished. Therefore, in the clinical assessment of patients at nutritional risk, tools such as the 'Malnutrition Universal Screening Tool' (MUST) is perhaps more useful and has gained favour in hospitals throughout the UK. In addition to BMI and percentage weight-loss, the effect of disease on food intake is taken into account and scored to categorise the patient's risk of malnutrition (BAPEN, 2012). Other tools for assessing anorexia include detailed food intake records or questionnaires such as the Functional Assessment of Anorexia/Cachexia Therapy scale (FAACT, (Chang et al., 2005)).

Anorexia has a neurohormonal origin due to disturbances of the central mechanisms controlling food intake along with several secondary mechanisms (Laviano et al., 2003) (Figure 1.1). Energy intake is controlled by the hypothalamus where peripheral signals convey information on energy and adiposity status. The arcuate nucleus within the hypothalamus plays a key role in feeding and metabolism. In particular, the pro-opiomelanocortin (POMC) neurons are a target for cytokine activation leading to hyperactivation of the melanocortin 4 receptor (MC4R) causing anorexia (Deboer and Marks, 2006, Marks et al., 2001). This has led to interest not only in developing MC4R antagonists to reverse anorexia, but also MC4R agonists in the treatment of obesity (Nahon, 2006).

The role of cytokines in cancer anorexia may well be effected through influencing both the neuropeptide Y (NPY) and POMC systems. In particular interleukin (IL)-1 has been associated with the induction of anorexia by blocking NPY induced feeding by a mechanism which may involve the attenuation of NPY activity by cytokines, inhibition of NPY synthesis, inhibition of neuronal firing rates or an attenuation of its postsynaptic effects (Plata-Salaman, 2000, King et al., 2000). An Australian study has suggested that macrophage inhibitory cytokine-1 (MIC-1), a cytokine known to be secreted by human tumours, is a potent modulator of appetite in mice bearing human prostate cancer xenografts (Johnen et al., 2007). This induced hypophagia due to reduced NPY expression and increased POMC expression within the arcuate nucleus. However, plasma MIC-1 levels only weakly correlated with dietary intake in patients with oesophago-gastric cancer. Furthermore, there was no correlation

FIGURE 1.1: PATHOGENESIS OF ANOREXIA



Abbreviations: Co-A – Coenzyme A, IL – interleukin, TNF – Tumour necrosis factor, NPY – Neuropeptide Y, AgRP – Agouti-related peptide, POMC – pro-opiomelanocortin, CART – cocaine and amphetamine-regulated transcript

with weight-loss, anthropometry or survival, questioning the relevance of MIC-1 in humans (Skipworth et al., 2010a).

Treatments to reverse muscle loss in cancer cachexia will involve addressing anorexia and attempting to improve any reduced food intake. Giving small, frequent energy-dense meals that are easy to eat (e.g. dairy products/ice cream) can improve food intake and patients should eat in pleasant surroundings and attention should be given to the presentation of food with avoidance of extremes of taste/smell and avoiding meals with a very high fat content which can delay gastric emptying (Stewart et al., 2006). The addition of nutritional supplements can help. Appetite stimulants such as megesterol acetate and corticosteroids can temporarily improve symptoms, but patients gain fat/oedema rather than muscle and there are potentially toxic side effects (Maltoni et al., 2001). At present, such medications are not licenced in the UK. More recently, the finding that cancer cachexia can alter appetite hormones such as ghrelin has led to trials looking at their potential in treatment of anorexia (Ashitani et al., 2009, Akamizu and Kangawa, 2011). However, results are inconclusive and as such, they have not yet been adopted into clinical practice (Blum et al., 2011).

1.2.3.3 FATIGUE

Fatigue is common in cancer patients (Fearon et al., 2006, Teunissen et al., 2007), and whilst the majority of cachectic individuals may suffer from fatigue, the ability of fatigue to define cachexia is more limited (Strasser, 2008). The perception of fatigue varies between individuals at markedly different rates making it quite a

subjective symptom and difficult to quantify. Physiologically, there are three types of fatigue – psychological (involving the central nervous system and patients' perception), muscular (resulting from adenosine triphosphate (ATP) depletion) and synaptic (occurring at the neuromuscular junction when the action potential frequency exceeds the rate of acetylcholine synthesis) (Seeley, 1995). In cancer cachexia, there is limited physiological research assessing fatigue according to these categories, with the majority of studies focusing on patients' feeling/assessment using questionnaires. Contributing factors can include anaemia, cytokine release, side effects of cancer therapies, as well as depression, anxiety and difficulty sleeping (Ahlberg et al., 2003). Assessment tools have been developed to investigate fatigue in cancer patients such as the FACIT (Functional Assessment of Chronic Illness Therapy; previously known as FACT-G (Functional Assessment of Cancer Therapy)) questionnaire. In pancreatic cancer patients with severe weight-loss, fatigue (assessed by FACIT) was found to be a significant symptom and furthermore, was predictive of survival (at least equalling predictive ability of KPS and Hb) (Robinson et al., 2008). Increased levels of cancer related fatigue (assessed by FACIT) have also been associated with lower handgrip strength, quadriceps strength and reduced muscle mass in patients with advanced gastrointestinal or lung cancers (Kilgour et al., 2010). Other groups have also associated increased fatigue with poorer measures of physical function (Brown et al., 2005, Stone et al., 2000). A recent review by Strasser et al (Strasser, 2008) concluded that fatigue was a global outcome of cachexia rather than a diagnostic marker, and other reports highlight the conflicting results as to whether there is an association between severe weight-loss and fatigue or depression (Blum et al., 2011). Given these inconclusive results, it remains to be

ascertained whether increasing fatigue is a global illness marker, caused by or leading to poorer muscle function and exacerbation of muscle loss.

1.2.3.4 OTHER EFFECTS

Along with the symptoms discussed above, there are a myriad of other symptoms that can affect patients with cancer cachexia. Many studies have demonstrated poorer QoL indices with cachexia (Fearon et al., 2006, Argiles et al., 2007, Fouladiun et al., 2007). The loss of weight and appetite in patients can have a negative psychological impact on patients and also their friends and family (Dodson et al., 2011). In particular, patients can feel distressed when family members focus on trying to increase their calorie/food intake in the context of anorexia and early satiety (Reid et al., 2009a, McClement, 2005). This can put strain on family relationships and the home environment along with causing patient anxiety about the possible outcomes of the disease (Reid et al., 2009a, Reid et al., 2009b, Strasser et al., 2006). Cachexia also has a detrimental impact on patients' body image (Rhondali et al., 2013, Fearon, 2008, Hinsley and Hughes, 2007), emotions, spirituality and relationships. Furthermore, patients often feel socially isolated which can be compounded by emotional distancing by carers and healthcare professionals (McClement, 2005).

1.2.4 **Impact of cachexia on muscle mass and function**

Weight-loss in cachectic individuals has been discussed in Section 1.2.3.1. This current section will focus specifically on the loss of skeletal muscle and changes in muscle function in cancer cachexia in keeping with the key theme of this thesis. The

molecular mechanisms underlying skeletal muscle depletion will be explored further in Section 1.2.6.

1.2.4.1 **Impact on muscle mass and methods of assessment**

Animal models of cachexia have been helpful both in gaining knowledge of molecular pathways relevant in muscle atrophy and in the assessment of subsequent changes in body composition by carcass dissection of cachectic animals at different points in the cachexia journey. Consistent with understanding of cancer cachexia and perhaps unsurprisingly, all animal models undergo profound muscle wasting (Bennani-Baiti and Walsh, 2011). However, observations in animal models are not necessarily directly reflective of what happens in patients (Bennani-Baiti and Walsh, 2011, Aulino et al., 2010). There exists a real challenge in assessing changes in muscle mass in human cancer cachexia, which may contribute to discrepant results between human and animal models of muscle loss. Along with the difficulty of measuring the true muscle compartment, any investigation of body composition when patients are diagnosed with cancer will only give the measure at one time point. Of course, muscle loss is a time dependant process, but very few (if any) patients will have an accurate measure of body composition when they were healthy pre-cancer diagnosis from which to derive true muscle loss. On top of this, there is great heterogeneity in body composition between individuals due to inherited factors and also choices influencing health behaviours (e.g. physical activity) that may cause difficulty in the interpretation of cross-sectional studies. For example, there will be patients who are motivated and strive to be physically active in spite of illness, whilst

at the other end of the spectrum are those who lead sedentary lives, are obese and don't participate in any physical activities.

In the past, changes in patient's weight alone, or alterations in anthropometry have been the methods employed in body composition studies. Even now, these measures are frequently used partly due to their ease of use in a wider population, lower financial outlay, and the difficulties of co-ordinating additional appointments for more detailed assessments and/or exposure of patients to radiation-based imaging. Indeed, a recent systemic review concluded that in clinical practice, muscle loss in patients with cancer cachexia can be assumed to relate to weight-loss provided the individual does not have fluid retention, a large tumour mass or obesity (defined as BMI $\geq$30 kg/m^2) (Blum et al., 2011).

With increasing availability of imaging modalities in parallel with advancements in technology, the opportunity for more detailed and sophisticated body composition studies has arisen. Cross-sectional imaging (using CT or MR imaging) has become the gold standard, but cost and sometimes access to scanners can be prohibitive. Other technologies to assess body composition such as dual energy X-ray absorptiometry (DEXA) and bio-impedance have been widely used by many research groups. Table 1.2 summarises some of the pros and cons of utilising each modality in body composition studies.

The loss of muscle mass (with or without loss of body fat) represents a cornerstone in each of the cachexia definition proposals (see Section 1.2.1). The rest of this section

Table 1.2: Pros and cons for different methods available for assessing body composition

	Pros	Cons
Anthropometry	Portable Well tolerated Inexpensive	Can be subjective Dependant on accurate land-marking No visualisation of anatomy
Bioimpedance	Portable Non-invasive Quick Relatively inexpensive	Influenced by oedema/recent physical activity No visualisation of anatomy
CT scan	Accurate anatomic outline of body tissues Quick	Requires appropriate facilities Dose of radiation (high) Expensive
DEXA	Well tolerated Relatively quick	Dose of radiation (low) No visualisation of anatomy
MR scan	Accurate anatomic outline of body tissues	Requires appropriate facilities Contraindications (e.g. pacemaker, claustrophobia) Expensive

This table is based on observations rather than direct comparison studies. The method that an individual investigator chooses will be influenced by local hospital/research centre equipment and funding available. Abbreviations: CT – Computed tomography, MR – Magnetic resonance, DEXA – Dual energy X-ray absorptiometry.

will thus focus on recent advancements in understanding of muscle mass in cancer cachexia related to the current gold standard of cross-sectional imaging.

CT scans have become a routine part of the clinical assessment and a method of monitoring treatment response in oncology patients. However, the potential for assessing body composition in cancer patients only came into popular use thanks to the work emanating from Professor VE Baracos' lab in Canada (Mourtzakis et al., 2008), which was based on earlier studies in healthy adults (Shen et al., 2004). Using image analysis software of a CT cross-sectional image at the 3rd lumbar vertebrae (L3) level, they were able to extrapolate whole body free fatty mass and appendicular skeletal muscle estimates, which correlated well with other body composition methods. In addition, CT could give information on individual muscles and viscera and differentiate between visceral, subcutaneous and intramuscular fat (Mourtzakis et al., 2008). Cut-offs were established to classify sarcopenic (in reference to low muscle mass rather than age-related muscle atrophy) and non-sarcopenic individuals. Other groups have used CT scans to measure different individual muscle/fat compartments (Bachmann et al., 2009), but the applicability to whole body measures and the association with survival has made Baracos' methodology more relevant to cancer cachexia.

Using this CT methodology, it has been shown that patients with oesophago-gastric cancer undergoing chemotherapy have a reduction in free fatty mass (FFM) and a greater proportion of patients become sarcopenic following completion of the treatment course (Awad et al., 2012). Furthermore, the toxic effect of

chemotherapeutic agents in different tumour types appears to be increased in those with lower muscle mass measures on CT (Prado et al., 2011, Prado et al., 2009, Prado et al., 2007). Likewise the apparent promotion of muscle gain by selumetinib in patients undergoing treatment for cholangiocarcinoma has been reported (Prado et al., 2012), opening up discussion about its potential as an anti-cachexia agent. These data would suggest that doses of chemotherapeutic agents should be adjusted in line with an individual patient's body composition and the degree of muscle wasting.

Another intriguing observation that has come to light with use of CT imaging, highlights the importance of muscle mass per se in cancer cachexia. The concept of sarcopenic obesity (i.e. 'hidden' muscle wasting - that despite a patients obese/overweight body habitus, they are deplete of muscle) was first publicised in studies of elderly people (Baumgartner, 2000, Zamboni et al., 2008). It has subsequently been estimated that 4-12% of elderly individuals are affected, using muscle mass as an indicator of sarcopenia (Stenholm et al., 2008). In patients with gastrointestinal and respiratory tumours, CT based cut-offs for sarcopenia demonstrated that 15% of obese cancer patients were sarcopenic which was associated with poorer functional status compared with obese non-sarcopenic patients and was also an independent predictor of survival (hazard ratio 4.2) (Prado et al., 2008). Interestingly, the authors also reported that sarcopenic obesity was more common in men than women, re-iterating the need for gender to be taken into consideration in studies of cancer cachexia. In another similar study of pancreatic cancer patients undergoing palliative treatment, 39.6% of patients were overweight/obese (BMI $\geq$25 kg/m^2) and of these, 40.9% were sarcopenic using CT

imaging. This sarcopenic overweight/obese cohort had the worst median survival of only 55 days and sarcopenic obesity was found to be an independent prognostic variable (Tan et al., 2009).

Whilst CT image analysis has proven useful for whole body muscularity measures, cross-sectional imaging of the thigh muscles has also been utilised in elderly and cancer patients with both CT (Maughan et al., 1983, Goodpaster et al., 2006, Goodpaster et al., 2008, Delmonico et al., 2009) and MR imaging (Weber et al., 2007b, Weber et al., 2007a, Weber et al., 2009). Assessing an individual muscle group such as the quadriceps carries the advantage of being able to relate these measures to muscle function of the same muscle group and thus calculate mechanical muscle quality (Maughan et al., 1983, Goodpaster et al., 2006, Weber et al., 2009). Additionally, in patients undergoing prolonged bed rest, lower limb musculature (e.g. the quadriceps) is prone to wasting (Pisot et al., 2008). Furthermore, antigravity muscle groups appear to respond differently to non-anti-gravity muscles (de Boer et al., 2008) and thus assessment of individual muscle groups in cancer cachexia may be more informative. Some groups have also utilised MR spectroscopy that details the chemical composition of muscle and gives information about, for example, intramyocellular lipid content (Weber et al., 2007b). Using MR imaging and MR spectroscopy, Weber et al were able to demonstrate a reduction in quadriceps cross-sectional area (CSA) of ~33% along with increased intramyocellular lipid content in cachectic gastrointestinal cancer patients compared with healthy controls (Weber et al., 2009, Weber et al., 2007a).

Advances in body composition analysis have been fundamental in improving our understanding of muscle mass and composition changes in cancer cachexia. Patients with low muscle mass have difficulty with activities of daily living, mobility and may end up bed bound which can further compound muscle loss. However, there must be additional factors that are relevant other than muscle mass alone. For example, endurance athletes and marathon runners may have relatively low muscle mass, but are obviously not unhealthy. Furthermore in ageing epidemiological research, there are reports that muscle strength is a stronger predictor of mortality compared with muscle mass (Newman et al., 2006). This raises the likelihood that, not only is the muscle mass relevant in cancer cachexia, but how well it functions and the relationship between the two (i.e. muscle quality) is important.

1.2.4.2 **Impact on muscle function and physical activity**

Given that muscle is one of the predominant tissues influenced by cancer cachexia, it is perhaps surprising to find that there are very few primary human studies assessing the impact on muscle function. A recent systemic review of symptoms relating to cancer cachexia identified only two studies investigating formally the relationship between reduced muscle strength and weight-loss in cancer patients (Blum et al., 2011). This seems illogical given the prominence that muscle function/strength has been given in the various proposals for the definition of cachexia (Section 1.2.1). Muscle mass has been correlated with strength in healthy adults (Maughan et al., 1983) and perhaps the assumption that the two are always highly correlated (Strasser, 2008) has distracted research groups from investigating this issue any further. Another contributing factor may be that extrapolation of elements from QoL scores

(e.g. physical function components from Short Form (SF)-36 health survey (SF-36) or European Organisation for Research and Treatment of Cancer (EORTC) QoL questionnaires) have been used as surrogates for direct physiological assessments of muscle function.

Muscle strength and physical activity have been shown to deteriorate in the presence of cachexia in animal models (Toledo et al., 2011, Baltgalvis et al., 2010, Baltgalvis et al., 2009, Aulino et al., 2010). However, the relationship between limb strength and cancer cachexia in humans is unclear. Increased fatigue has been related to poorer muscle strength in palliative lung/gastrointestinal cancer patients (Kilgour et al., 2010) and a study in gastrointestinal cancer patients demonstrated a reduction in absolute quadriceps strength of 30-40% (Weber et al., 2009). Contrary to this, a study in lung cancer patients showed similar quadriceps strength compared with healthy controls (Wilcock et al., 2008). One explanation is that there may be differences in functional changes according to tumour type. However, whilst the lung cancer study recruited patients with weight-loss <10%, (Wilcock et al., 2008) patients in the gastrointestinal cancer study (Weber et al., 2009) all had weight-loss >10% in 6 months. It is thus more likely that quadriceps muscle function is influenced by the degree of weight-loss. Interestingly, there are data from both animals (Aulino et al., 2010) and patients with gastrointestinal cancer (Weber et al., 2009) that report normalising strength to muscle size/CSA (muscle mechanical quality) abrogates the significant difference from controls i.e. that the available strength per unit muscle is not reduced in cachexia. In contrast, animal models have suggested that the process of cachexia involves selective and early loss of key

elements of the contractile structures within skeletal muscle which would likely impact on muscle mechanical quality (Acharyya et al., 2005). Moreover, ultrastructural analysis has demonstrated myofibrillar disarray, irregular muscle membranes and dystrophic muscle morphology in the muscles of tumour-bearing cachectic animals (Acharyya et al., 2005). Given these contrasts, there is a pressing need to explore these relationships in other human datasets.

Hand grip dynamometry has been shown as a sensitive marker of sarcopenia in elderly adults (Lauretani et al., 2003) and is suggested as the preferred measure of muscle strength in studies of cancer cachexia (Fearon et al., 2011) mainly due to the accessibility and ease of measurement in the clinical setting. Patients with low hand grip strength prior to surgery for oesophageal cancer suffer more morbidity and have increased post-operative mortality rates (Chen et al., 2011) and hand grip strength has been demonstrated to be significantly lower in cachectic pancreatic cancer patients with weight-loss >10% versus 5-10% (Fearon et al., 2006).

The use of physical activity monitoring with portable accelerometers is well tolerated by cancer cachexia patients (Maddocks et al., 2010) and is thought to represent a useful objective endpoint for trials (Maddocks et al., 2010, Dahele et al., 2007, Strasser, 2008). One study, utilising the ActiGraph physical activity monitor (accelerometer), in palliative pancreatic cancer patients demonstrated significantly reduced physical activity in weight losing cancer patients versus healthy volunteers. In addition, cancer patients had decreasing spontaneous physical activity associated with progression of their disease with time (Fouladiun et al., 2007). Weight-loss

(along with plasma CRP and Hb) was a significant predictor of spontaneous activity and SF-36 self-reported physical functioning and bodily pain predicted variations in overall daily physical activity (Fouladiun et al., 2007). Similarly in a study of patients with UGI malignancies undergoing palliative care, using the ActivPAL^{TM} accelerometer a reduction in the median total number of steps taken by cancer patients of 43% compared with controls was seen along with a fall in the time spent in quiet standing of 32% (~2hrs/day less) (Dahele et al., 2007). This tendency towards sedentary behaviour and reduced physical activity will undoubtedly exacerbate decline in muscle mass and function in cachectic cancer patients given the data from bed-rest and immobility studies (Biolo et al., 2005).

These issues clearly need to be explored further given that the maintenance of independence and QoL are desirable by patients and that physical activity relates to psychological wellbeing (Maddocks et al., 2010, Netz et al., 2005, Rejeski and Mihalko, 2001, McAuley et al., 2000).

1.2.5 **Impact of cachexia on survival**

In 1932, Shields Warren from Harvard medical school reported a series of 500 autopsies on cancer patients (Warren, 1932). The immediate cause of death in 114 of these (22%) was secondary to inanition of the patients and up to two thirds of this cadre of patients exhibited some degree of cachexia (Palesty and Dudrick, 2003). In 1980, Dewys et al demonstrated the adverse effects of weight-loss on survival and introduced the notion that even lower amounts of weight-loss (<5%) may significantly worsen prognosis (it had previously been thought that only >10% was a

'significant' weight-loss) (Dewys et al., 1980). There has already been discussion relating to the poorer survival seen in patients with cancer cachexia with relation to weight-loss earlier in the introduction to this thesis (Section 1.2.3.1). This section will thus discuss various scoring systems that have been proposed in recent years.

1.2.5.1 SCORING SYSTEMS

In the field of oncology, where survival has traditionally been used as the gold-standard outcome measure, prognostic scoring systems have received a lot of interest. The ability to make informed decisions about treatment options and have frank discussions with patients and their relatives about disease trajectory are made much easier if there is some way of reliably predicting a patient's clinical course. Likewise, although individual markers have been associated with morbidity and mortality in cachectic patients, there has been growing interest in developing prognostic scoring systems combining more than one measure in order to more accurately predict survival and outcome.

(i) Glasgow prognostic score (GPS)

This simple scoring system is based on CRP and albumin levels. A point is awarded for a raised CRP or hypoalbuminaemia to give a maximum score of 2 if both are present. Prognosis has been shown to be significantly poorer in patients with metastatic breast or non-small cell lung cancer and a higher GPS score (Forrest et al., 2004, Al Murri et al., 2006). GPS has also been associated with changes in several biochemical variables associated with systemic inflammation in patients with advanced lung and gastrointestinal cancer (Brown et al., 2007), and more recently

has been correlated with concentration of proinflammatory cytokines (IL1, IL6, IL8, TNF-α, VEGF-a, midkine) and transferrin (Krzystek-Korpacka et al., 2008).

(ii) CRP plus white blood cell (WBC) count

A group in Montreal used a similar scoring system to the GPS, but replaced albumin with WBC count. In a study of patients with non-small cell lung cancer, median survival was only 3 months in those with a score of 2 at presentation, compared with 7 months if patients scored 1 and 19 months in those who scored 0 (MacDonald, 2007).

(iii) CRP, weight-loss and food intake

In a study of 170 weight-losing patients with unresectable pancreatic cancer, weight-loss alone was not a prognostic variable and did not identify patients with altered body composition or reduced subjective function or health status. However, the three-factor profile of systemic inflammation (CRP ≥ 10mg/l), reduced food intake (≤ 1500kcal/day) and weight-loss (≥10%) defined those with both adverse function and prognosis (Fearon et al., 2006).

(iv) Clinical prognostic scoring system

This scoring system was developed in an effort to improve prognostic accuracy and risk predication of death in patients with gastro-oesophageal cancer. Variables used for scoring are CRP, rate of weight-loss, KPS and clinical stage of disease (primary Tumour, lymph Nodes and presence of Metastases (TNM) staging system). Analysis of 220 patients revealed accurate predication of death at 12 and 24 months (Deans et

al., 2007b). Weight-loss >2.75% per month was an independent prognostic indicator of decreased survival in oesophageal cancer.

1.2.6 **Molecular mechanisms of muscle wasting in cachexia**

Although there may be loss of both fat and muscle tissue in cancer cachexia, it is the loss of skeletal muscle that will have the most profound effects on patients' function and activities of daily living. Skeletal muscle is essential to provide movement, strength, respiration, balance, posture, and to regulate body temperature (Lenk et al., 2010). There is some recent evidence of cross-talk between fat and muscle and that the former may play a key role in modulating muscle wasting (Section 1.2.3.1). However, the main theme of this thesis is focussed on skeletal muscle in cachexia, and thus this section will concentrate on molecular mechanisms of atrophy within skeletal muscle. Potential biomarkers of cachexia could be either systemic mediators of the cachectic process or relevant end-organ change within skeletal muscle that occur as a result of such mediator activity. This section provides an overview of the structure and composition of skeletal muscle and physiology, a summary of known systemic mediators of cachexia followed by a focus on the molecular pathways and alterations in structural proteins relevant to muscle wasting in cachexia. Due to the lack of data in some of these areas, the discussion includes knowledge derived from other models of muscle wasting where relevant.

1.2.6.1 **Structure and functional physiology of skeletal muscle**

1.2.6.1.1 Gross anatomy of muscle

Muscle is defined as:

" an animal tissue by contraction of which bodily movement is effected" from the Latin musculus, the diminutive of mus meaning (a little) mouse.
(Chambers Etymological English Dictionary, 1960)

There are two major types of muscle in man – smooth and striated. Smooth muscle is an involuntary muscle (meaning it is controlled by the autonomic nervous system) and is primarily responsible for contractions in hollow organs such as blood vessels, the gastrointestinal tract, the bladder, glands and eyes. Striated muscle can be either cardiac (a highly specialised involuntary muscle) or skeletal (under voluntary control) (Seeley, 1995). Cardiac and smooth muscle do not form part of the investigation of this thesis and thus subsequent discussion in this section will relate exclusively to skeletal muscle.

Skeletal muscle is the major reservoir of body proteins, consisting of around 640 separate muscles that account for 40% of total body weight or 50% of fat free mass (FFM). The majority of muscles are classified (according to the structural arrangement) as fusiform with muscle fibres arranged longitudinally tethered by tendons at each end. Alternatively, muscles can be pennate with the muscle fibres inserting into a tendon at an angle. Muscles are arranged in bundles of fibres (~85% of total tissue) bound by connective tissue mainly comprised of collagen. The myofibril is the base unit of muscle comprising thick (predominantly myosin) and thin (predominantly actin) filaments. Other organelles such as mitochondria are

packed between the myofibrils. Collections of myofibrils are bound by a delicate external lamina and the sarcolemma to make up muscle fibres that are surrounded by the endomysium. Muscle nuclei lie underneath the sarcolemma. A heavier connective tissue layer called the perimysium encases bundles of muscle fibres into fascicles, which in turn are arranged into groups ensheathed by the epimysium. Each muscle is finally encased in fascia (Seeley, 1995) (Figure 1.2).

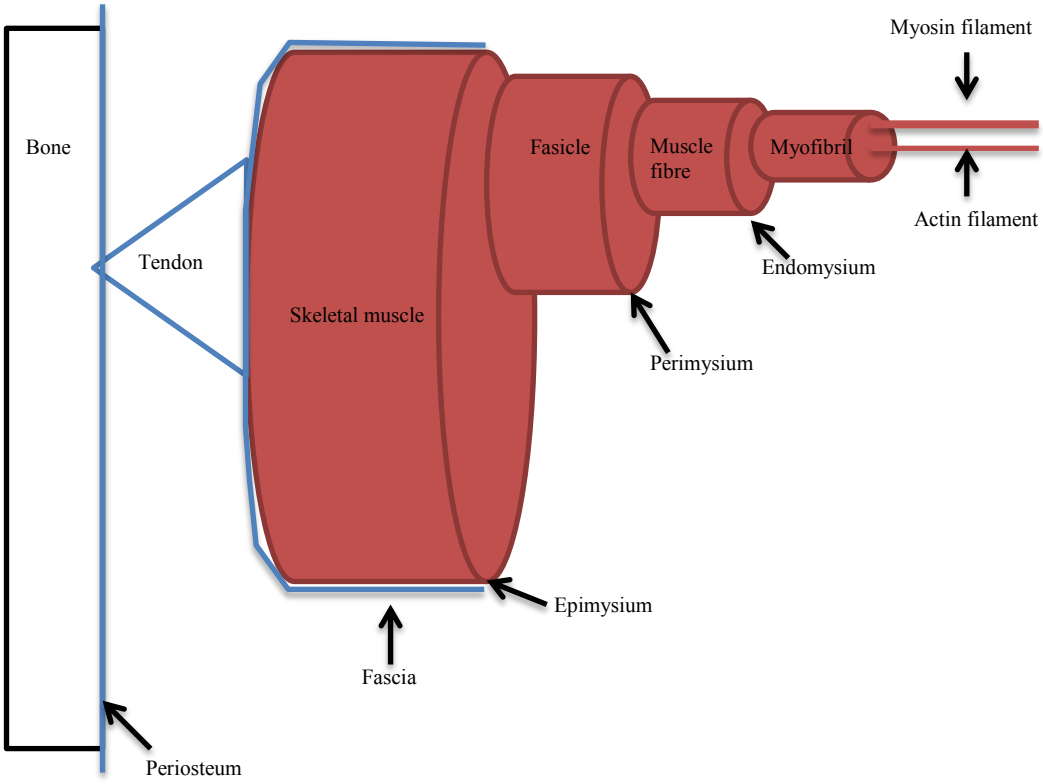
MUSCLE ULTRASTRUCTURE

THE MYOFIBRIL

There are three classes of muscle protein as outlined in Table 1.4 (Goll et al., 2008).

The myofibrillar proteins are integral to muscle contraction and function and are thought to be key targets in cancer cachexia (Acharyya et al., 2004). Myofibrils are 1-3 μm in diameter and are made up of myofilaments. There are more than 17 different proteins present in the myofibrillar structure (Goll et al., 2008). Actin is the primary protein within thin filaments, which measure 8 nm in diameter and 1000 nm in length. Thick filaments are predominantly composed of myosin and are 12 nm in diameter and 1800 nm in length. These filaments are specifically organised into repetitive sarcomere subunits that give rise to the typical 'striated' appearance of skeletal muscle under the microscope (Figure 1.3A). Two other filament components contribute to the sarcomere – one spans half-sarcomeres and is composed of single molecules of titin (the largest vertebrate protein) and the other

FIGURE 1.2: ARRANGEMENT OF SKELETAL MUSCLE



Schematic illustrating the composition and arrangement of individual muscles. Each muscle is made up of highly organised groups of muscle fibres, which in turn are composed of myofilaments (actin and myosin filaments).

Table 1.3: Classes of skeletal muscle protein

	Myofibrillar	Sarcoplasmic	Stroma
% Muscle protein by weight	55-60	30-35	10-15
Roles	Structural Muscle function	Enzymes	Collagen Extracellular matrix

Percentage of the individual muscle protein classes and their roles within skeletal muscle.

filament is made from nebulin that spans the length of the actin filaments (Clark et al., 2002).

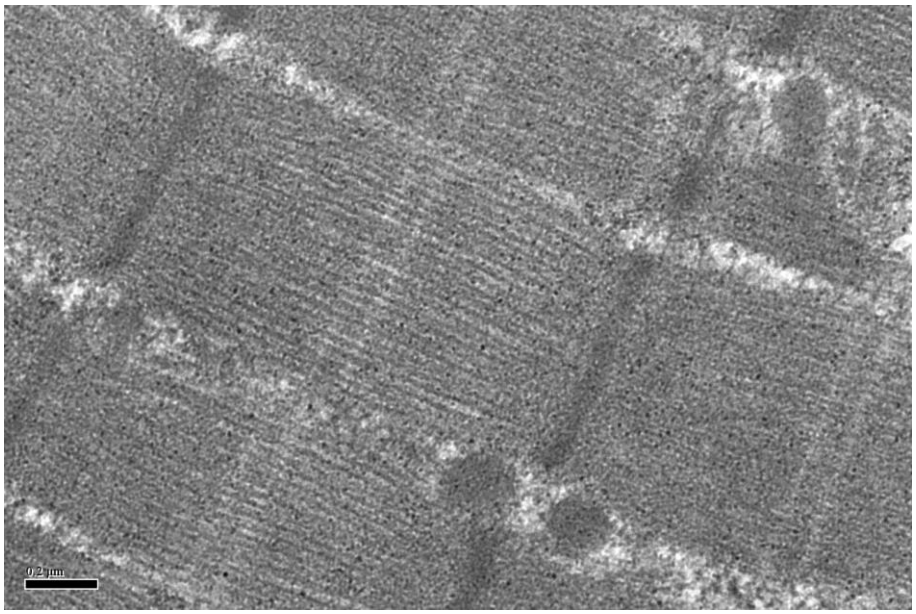
THE SARCOMERE

The sarcomere is divided into zones, bands and discs according to its appearance under a microscope (Figure 1.3B):

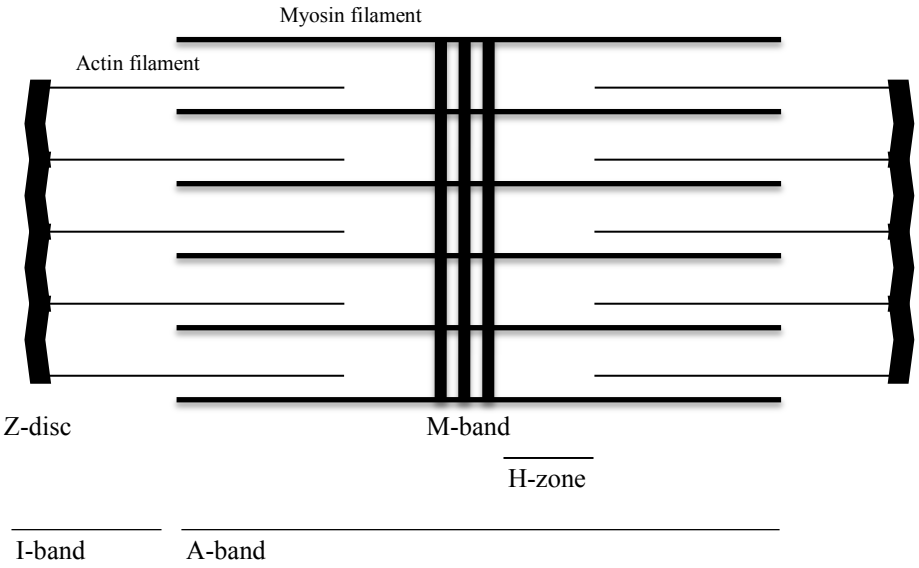
- Z-disc – from the German zwischen meaning between, it is a structure for the attachment of actin myofilaments
- A-band – stands for anisotropic, dark band extends the length of the myosin myofilaments within a sarcomere, the actin and myosin myofilaments overlap for some distance at both ends of the A band and on cross-section of this overlap, each myosin myofilament is surrounded by six actin filaments
- I-band – stands for isotropic, light band includes a Z-disc and extend from either side of the Z-disc to the ends of the myosin myofilaments, the I band on either side of the Z-disc consists only of actin myofilaments
- H-zone – from the German helle meaning bright, within the A band centrally where actin and myosin myofilaments do not overlay and only myosin myofilaments are present
- M-band – from the German mittel meaning middle, a dark band in the middle of the H zone, consisting of delicate filaments that attach to the centre of myosin myofilaments
- T-tubule – tube-like invagination along the sarcolemma where actin myofilaments and myosin myofilaments overlap

FIGURE 1.3: THE SARCOMERE

A.



B.



*(**A.**) Electron microscopic image (11000x magnification) illustrating the typical striated appearance of skeletal muscle caused by the repetitive sarcomere units. The ruler at the bottom left corner of the image represents 0.2 µm. (**B.**) Diagram outlining the individual zones, bands and discs that can be seen in A. A single sarcomere extending from one Z-disc to another is illustrated.*

FIBER TYPES

In humans, muscle fibres have been classified into two main types based on whether they express the gene for a slow or a fast myosin isoenzyme: slow type I/oxidative fibres which have a red appearance owing to the high capillary density, and fast type II/glycolytic fibres which are paler/white in appearance.

In cachexia, evidence from animal models suggests that type II fibres are selectively targeted (Acharyya et al., 2005) with relative preservation of type I fibres in fasting (Li and Goldberg, 1976), exposure to glucocorticoids (Goldberg and Goodman, 1969, Dahlmann et al., 1986), sepsis (Tiao et al., 1997), and cancer cachexia (Baracos et al., 1995, Acharyya et al., 2004). However, whether this occurs in human cancer cachexia is unclear. Furthermore, not all groups have demonstrated type I and II fibre differences even in animals. Indeed in a recent exposition of the colon-26 cachectic murine model, both glycolytic and oxidative fibres underwent wasting (Aulino et al., 2010).

The activity patterns of a muscle do seem key in determining its phenotype. If muscle cells are infrequently recruited, they develop into fast/glycolytic units whereas if they are recruited more often, they form slow/oxidative units. In the colon-26 murine model of cancer cachexia, there have been reports of switching of myosin isoforms in the soleus muscle of cachectic mice (Diffee et al., 2002), although in pancreatic cancer patients with cachexia, no difference in the ratio of fast/slow myosin isoform was demonstrated compared with controls (Schmitt et al., 2007).

1.2.6.1.2 MUSCLE PHYSIOLOGY:

CONTRACTION AND THE SLIDING FILAMENT THEORY

Skeletal muscle contraction is a voluntary process, although can be involuntary by the stimulation of reflexes. Despite the macroscopic appearance of the muscle reducing in size during contraction, the myofilaments do not actually shorten. Rather, a phenomenon referred to as the sliding-filament theory occurs; where myofilaments slide past one another to cause the sarcomere to shorten (Seeley, 1995, Berne, 1996). During contraction, cross-bridges form and release between the thin (actin molecules) and thick (the heads of the myosin molecules) filaments. This occurs in a stepwise fashion with the result that the thin and thick filaments appear to be sliding over one another until at full contraction the I-band and H-zone completely disappear. This cross-bridge cycling accounts for most of the body's ATP (energy) consumption. The release of cross-bridges results in relaxation of muscle and sarcomeres passively (although usually with application of some force) lengthen with the resultant reappearance of the I-band and H-zone.

A muscle contraction is initiated following elicitation of an action potential in a motor nerve. Each nerve controls many muscle cells that are functionally grouped into a 'motor unit' although each muscle cell will only have one neuromuscular junction. Synchronous contraction of a motor unit occurs due to the generation of action potentials in all muscle cells triggered by the release of acetylcholine at endplates.

The sliding-filament theory is possible due to a process termed excitation-contraction coupling (Berne, 1996). This has four main steps:

- action potential induced by acetylcholine that leads to;
- opening of calcium channels in the sarcoplasmic reticulum
- subsequent diffusion of calcium which reversibly binds to troponin
- finally this causes conformational alteration of the thin filament allowing cross-bridge cycling to commence

The binding of calcium to troponin (which has 4 calcium binding sites) is one of the key steps in this process. It is attached to actin filaments and has the unique capability of changing its shape when calcium is bound.

This section has given an overview of the anatomy and physiology of healthy skeletal muscle. The next section will discuss the systemic and intracellular mechanisms that are thought relevant in muscle wasting related to cachexia.

1.2.6.2 **Systemic mediators of cachexia**

Cancer cachexia is considered, at least in part, to result from interactions between the host and the tumour. Components of this interaction are then thought to drive molecular pathways resulting in anorexia and muscle atrophy. This can occur either directly (such as with proteolysis-inducing factor (PIF) stimulating the ubiquitin proteasome pathway (UPP)), or indirectly via other cascades (such as pro-inflammatory cytokines leading to induction of the acute phase response (APR)). There are also various hormones that have been implicated in muscle wasting. This section gives a brief overview of the potential systemic mediators of cancer cachexia.

1.2.6.2.1 **Tumour-derived mediators**

PIF

PIF is a 24 kDa sulphated glycoprotein. It was originally discovered in the murine adenocarcinoma (MAC)-16 cachectic mouse model (Todorov et al., 1996) but has also been isolated from the urine of cachectic cancer patients (Cariuk et al., 1997). PIF appears to both depress protein synthesis and increase protein degradation (both by ~50%) (Lorite et al., 1998). Its potential actions are:

- stimulation of the UPP (Lorite et al., 2001)
- activation of the transcription factor Nuclear Factor Kappa-B (NFκB), with resultant specific depletion of myosin (Wyke and Tisdale, 2005)
- increase tripeptidyl peptidase II which cleaves peptides generated by the proteasome into tripeptides (Balow et al., 1986, Chand et al., 2005)
- induction of cytokine production (IL-6, IL-8 and TNFα (Watchorn et al., 2002, Watchorn et al., 2005)).

Whilst all of these mechanisms are relevant to cachexia, other research groups have been unable to identify PIF or relate it to cachexia (Jatoi et al., 2006). As such, the exact role and relevance to human cancer cachexia remains under debate (Wieland et al., 2007, Blum et al., 2011).

Lipid mobilising factor (LMF)/ Zinc-α2-glycoprotein (ZAG)

LMF (homologous to ZAG (Hirai et al., 1998)) is produced by cachexia inducing tumours and has been shown to stimulate lipolysis in both animal models and in

cachectic cancer patients (Mracek et al., 2011). ZAG expression and secretion by adipose tissue is enhanced in cachectic cancer patients. Given its lipid mobilising effect, ZAG may contribute to adipose atrophy associated with cancer cachexia in human beings (Mracek et al., 2011). Although interest has generally focussed on its effects on lipid mobilisation, substrate utilisation and activation of mitochondrial oxidative pathways in brown adipose tissue, there is also some evidence that there may be an effect on skeletal muscle. LMF/ZAG acts through a β-adrenoreceptor and since β-agonists (e.g. formoterol) stimulate skeletal muscle hypertrophy in animals, it is not surprising that LMF has been shown to stimulate protein synthesis and decrease protein degradation (via reduction in UPP) (Islam-Ali and Tisdale, 2001). Furthermore, formoterol has been shown to reverse muscle wasting in tumour bearing rats by decreasing protein degradation (mainly via inhibition of UPP) and increasing protein synthesis (Busquets et al., 2004) suggesting the LMF/ZAG may protect skeletal muscle from atrophy and explain why loss of fat precedes loss of protein in cachectic cancer patients. More recently, another lipid mobilising factor, distinct from ZAG, has been shown to promote lipolysis in the methylcholanthrene (MCA)-induced sarcoma bearing cachexia model (Argiles et al., 2007).

Cytokines and the APR

(i) 'Classical' cachectic pro-inflammatory cytokines

Pro-inflammatory cytokines, in particular IL-6, IL-1, TNF and interferon (IFN)-γ, are central to systemic inflammation and the induction of the APR (Table 1.4). Cytokine interaction between host and tumour cells within the tumour mass results in activation of peripheral blood mononuclear cells (PBMC) passing through the local

Table 1.4: Main pro-inflammatory cytokines

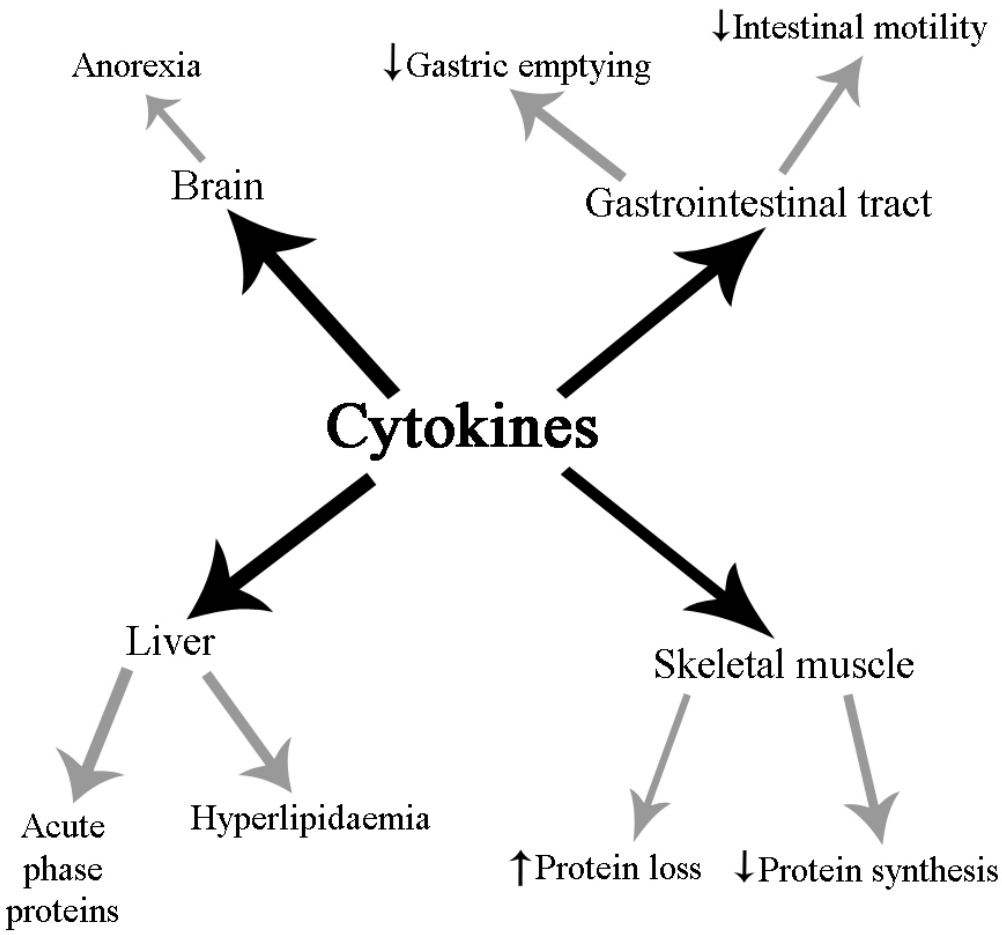
Cytokine	Cellular source	Functions
TNFα	Macrophages	Mediates muscle protein loss
	Lymphocytes	Stimulates liver lipogenesis
		Insulin resistance
		Anorexia
IL-1β	Macrophages	Induces liver acute phase proteins
		Fever
		Arthralgia
		Myalgia
		Anorexia
IL-6	Macrophages	Main inducer of acute phase response
	Fibroblasts	(in combination with other mediators)
	Mast cells	Suppresses food intake
	T cells	
IFN-γ	Lymphocytes	Potentiates catabolic effects of other mediators
	Natural killer cells	

The cellular source of the main pro-inflammatory 'classical' cytokines and an outline of some functions relevant in the development of cachexia and the acute phase response. Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

tumour vasculature. Increased pro-inflammatory cytokine release by PBMCs has been demonstrated previously in patients with evidence of an APR (Wigmore et al., 2002, O'Riordain et al., 1999). Recent advances in tumour biology have suggested that macrophages may be one important cellular sub-type involved in the intra-tumoural interaction between host and tumour cells (Yuan et al., 2008). In cancer, host mononuclear cells are recruited to tumours by various signals including hypoxia. Thus, a major inflammatory component of tumour stroma is made up of so-called tumour–associated macrophages (TAMs). The resultant Th-2 type micro-environment may favour tumour progression via the promotion of angiogenesis, the remodelling of the extracellular matrix to allow invasion, and the suppression of adaptive immunity (Yuan et al., 2008). How TAMs may be involved in the activation of host PBMCs is not yet known but macrophage-associated cytokines have been shown to induce an APR in isolated human hepatocytes (Wheelhouse et al., 2006). Furthermore, high tumour chronic inflammatory cell content is associated with poor patient prognosis (Deans et al., 2006). Once activated, host PBMCs then initiate/trigger their own cytokine cascade. Circulation of PBMCs and cytokines to target end organs may then induce various distant effects (Figure 1.4).

In the liver, during a positive APR, net export protein synthesis is increased. Near complete inhibition of the ability of human hepatocytes co-cultured with PBMC's to produce acute phase protein has been achieved by using anti-IL-6 antibodies (O'Riordain et al., 1999). This implies that IL-6 is one of the key mediators of this response. In mice with a mutation in the adenomatous polyposis coli (APC) tumour suppressor gene, elevated circulating IL-6 levels are associated with the presence of

FIGURE 1.4: DISTANT EFFECTS OF CYTOKINES



cachexia (Baltgalvis et al., 2008). Knockout of IL-6 prevented loss of muscle weight and epididymal fat, and reduced intestinal polyp number by 32%, implying the existence of an IL-6 cytokine amplification loop between host and tumour cells. Furthermore, recent human studies have suggested that IL-6 overexpression in weight losing pancreatic cancer patients is related to the ability of certain IL-6-producing tumours to sensitise PBMC and induce cytokine expression in PBMCs (Martignoni et al., 2005). However, there are reports that other pro-inflammatory markers such as IL-1β may be more closely associated with clinical features of cancer cachexia (Scheede-Bergdahl et al., 2012).

In the brain, pro-inflammatory cytokines induce central anorexia by exerting actions on the orexigenic NPY and the anorexigenic POMC systems within the hypothalamic arcuate nucleus, thus resulting in reduced food intake. In particular, IL-1 may block feeding induced NPY, possibly via inhibition of NPY synthesis, inhibition of neuronal firing or an attenuation of its post-synaptic effects (Plata-Salaman, 2000, King et al., 2000). A recent Australian study has suggested that MIC-1, a cytokine know to be secreted by human tumours, is a potent modulator of appetite in mice bearing human prostate cancer xenografts (Johnen et al., 2007). The associated hypophagia appeared to be caused by reduced NPY expression and increased POMC expression within the arcuate nucleus. Furthermore, a MIC-1 antibody could reverse weight, fat and lean tissue loss.

In skeletal muscle, in vitro and in vivo studies have confirmed a wide range of intracellular effects of cytokines that result in the net degradation of myofibrillar

protein and amino acid mobilisation. These include the promotion of insulin resistance, activation of NFκB transcription factor pathways, and increased activity of the UPP. Studies in C2C12 myoblasts and murine models have suggested that pro-inflammatory cytokines may target depletion of certain myofibrillar proteins in a specific fashion: administration of TNF and IFN-γ reduced mRNA and protein expression of myosin heavy chain (MyHC) but not other core proteins (Acharyya et al., 2004). Further studies by the same American group, using the colon-26 mouse, a model believed largely to be cytokine dependent, have also suggested that pro-inflammatory cytokines may disturb the membranous morphology of skeletal muscle (Acharyya et al., 2005). Such mice demonstrated significant deregulation of the dystrophin glycoprotein complex (DGC), a large membrane-bound complex involved in the protection of muscle from contraction-induced injuries. Furthermore, deregulation of the DGC was associated with enhanced cancer-induced muscle atrophy.

(ii) Other pro-inflammatory cytokines

More recently, there has been growing interest in the role of other pro-inflammatory cytokines in cancer cachexia. IL-8, vascular endothelial growth factor (VEGF)-a, and midkine have been implicated in recent years. These cytokines are involved in stimulating the expression of other pro-inflammatory agents, increasing the permeability of blood vessels and triggering degranulation of inflammatory cells (Kadomatsu and Muramatsu, 2004, Henson and Vandivier, 2006, Dvorak, 2005). IL-8 has been shown to modulate acute phase protein production from isolated human hepatocytes (Wigmore et al., 1997a). More recently, a group from Poland studied

levels of VEGF-A, VEGF-C, IL-8, midkine, TNFα, IFN-γ, IL-1 and IL-6 in the serum of 96 gastro-oesophageal cancer patients (Krzystek-Korpacka et al., 2008), 49 of whom were cachectic (as defined by weight-loss >5% in preceding 3 months). There was a significant increase in both IL-6 and IL-8 levels in cachectic versus non-cachectic patients and a trend to raised levels of midkine and VEGF-a. Using multiple logistic regression modelling, both midkine and VEGF-a were independent predictors of weight-loss in gastro-oesophageal cancer patients.

(iii) Anti-inflammatory cytokines

Whilst pro-inflammatory cytokines induce cachexia and the APR, other cytokines (such as IL-4, IL-10 and IL-13) may have anti-inflammatory actions and may therefore be potential repressors of cachexia. The development of wasting and the APR in the cachectic patient may thus depend on the balance between pro- and anti-inflammatory cytokines. Both IL-4 and IL-10 reduce the production of the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNFα (Fiorentino et al., 1991, Bogdan et al., 1991, de Waal Malefyt et al., 1991). This has led to investigation into the potential for using anti-inflammatory cytokines in the treatment of cancer cachexia. In the colon-26 adenocarcinoma mouse model, IL-10 gene transfer reduces cachexia and prolongs survival (Fujiki et al., 1997). IL-10 gene transfer has also been shown to suppress the peritoneal dissemination of gastric cancer cells in nude mice. Improved survival and a reduction in weight-loss were also observed in the IL-10 treated group (Tanaka et al., 2008).

(iv) Host Cytokine Genotype

In recent years, host genotype has been proposed as one determinant of the development of cachexia. In particular, cytokine genotype may influence the aetiology of systemic inflammation and the APR (Tan et al., 2008). Specific IL-10 and IL-6 polymorphisms have been related to systemic inflammation and adverse prognosis, and a TNFα polymorphism was associated with adverse prognosis in patients with gastro-oesophageal cancer (Deans et al., 2007a). IL-1β polymorphisms were associated with development of cachexia and adverse prognosis in pancreatic and gastric cancer (Barber et al., 2000b, Zhang et al., 2007). Conversely, the IFNγ 'allele 2' polymorphism was related to improved survival in patients with non-resectable pancreatic cancer (Halma et al., 2004).

1.2.6.2.2 **Host-derived mediators**

The APR in Cancer Cachexia

An organism responds to the presence of infection, tumour, immunological disorders, tissue injury, trauma or surgery by eliciting the APR, a response designed to help limit tissue injury. However, in certain circumstances when the APR is prolonged or severe, it can be lead to detrimental effects. The APR is a complex physiological event involving reprioritization of hepatic protein synthesis, resulting in increased synthesis of the positive acute phase proteins (serum amyloid A, CRP, fibrinogen, α1-acid glycoprotein, α1-antichymotrypsin and haptoglobin). Furthermore, the plasma concentrations of negative acute phase proteins (albumin, per-albumin and transferrin) fall. However, reduction in negative acute phase reactants is not due to decreased hepatic synthesis, but possibly by increased transcapillary escape

secondary to an increase in micro-vascular permeability. This was demonstrated by the finding that total albumin synthesis rate in both the fasting and fed state is not different between patients with advanced cancer and healthy controls, even in the face of significant hypoalbuminaemia (Fearon et al., 1998, Barber et al., 2000a). This means that, in the cachectic patient, the increased hepatic synthesis of positive acute phase reactants is not compensated for by reduced synthesis of negative acute phase reactants. Thus, the net result is increased demand for amino acids by the liver.

How the APR is linked to the development of muscle atrophy in cancer cachexia is not clear. However, it has been hypothesised that persistent hepatic synthesis of positive acute phase reactants represents a nutritional "sink" into which amino acids mobilised from skeletal muscle are lost. The significant mismatch between the amino-acid composition of muscle and acute phase proteins (it has been calculated that 2.6g of muscle protein must be catabolised to produce 1g of fibrinogen) (Preston et al., 1998, Reeds and Hutchens, 1994) demonstrates how aggressively the APR may drive nutritional depletion. To complicate matters further, nutritional support, an obvious required treatment for malnourished cancer patients, may accelerate positive acute phase protein synthesis and thus exacerbate one of the basic mechanisms that contributes to the loss of lean tissue. In weight losing patients with pancreatic cancer, synthesis rates of hepatic export proteins (fibrinogen) are elevated in the fasted state, but rise even higher during enteral feeding (Barber et al., 2000a). These findings emphasise the need for inclusion of anti-inflammatory/anti-APR supplementation within any programme of high calorie/high protein nutritional support in the treatment of cancer cachexia. After a 3-week course of oral nutritional supplements

enriched with eicosapentaenoic acid (an anti-inflammatory n3 fatty acid) in patients with pancreatic cancer, the combined synthetic rate of albumin and fibrinogen was significantly reduced in the fed state and demonstrated a tendency to fall in the fasting state. Furthermore, patient weight had stabilised (Barber et al., 2004).

The Relationship Between APR and Survival

The positive acute phase reactant CRP is often used as a marker of the APR and can be measured easily in the clinical setting. In cancer patients, CRP correlates with the circulating concentration of IL-6 (Barber et al., 1999) and is thus an indirect marker of systemic inflammation and pro-inflammatory cytokine activity. CRP has gained a great deal of interest in recent years as a prognostic marker in cancer patients. In several tumour types (lung (Forrest et al., 2004, Scott et al., 2002), lymphoma (Legouffe et al., 1998), breast (Heys et al., 1998), renal (Bromwich et al., 2004), pancreatic (Falconer et al., 1995), gastro-oesophageal (Ikeda et al., 2003, Rashid et al., 1982, Crumley et al., 2006) and colorectal (McMillan et al., 2003)), an elevated CRP is associated with an adverse outcome and poor prognosis. Indeed, in gastro-oesophageal cancer, it has been used to predict the outcome of patients post tumour resection. In 67 patients with resectable gastric or oesophageal cancer, CRP in isolation has been shown to be more accurate than the tumour stage in predicting early prognosis one year after surgical resection (Deans and Wigmore, 2005). Pre-operative elevation of CRP has also been associated with reduced cancer specific survival following curative resection of colorectal cancer (McMillan et al., 2003), and in a study of 102 patients with pancreatic cancer, the presence of a high CRP reduced median survival from 222 days to just 66 days (Falconer et al., 1995).

Fibrinogen, another positive acute phase reactant, has been studied to a lesser extent than CRP. Investigations in melanoma and lung cancer patients have shown an association between elevated fibrinogen levels and a poor outcome (Guida et al., 2003, Pavey et al., 2001). Another study of 212 lung cancer patients at the Royal Marsden Hospital who were taking part in phase I trials and also a retrospective study of 172 non-small cell lung cancer (NSCLC) patients revealed low levels of albumin as a prognostic factor for reduced survival (Arkenau et al., 2008, Paralkar et al., 2008).

1.2.6.2.3 **Other circulating mediators**

Calcium and Vitamin D

A potential role for calcium and vitamin D in muscle wasting relates to evidence from the myopathy (predominantly Type II fibre atrophy) seen in osteomalacia, which can be exacerbated in ageing (Solomon and Bouloux, 2006, Janssen et al., 2002). Calcium, phosphate and vitamin D levels all impact on muscle function. Smaller muscle fibres are seen in vitamin D null mice and supplementation can reverse these changes (Endo et al., 2003). In sarcopenia, patients with low vitamin D have been shown to have ~2x risk of reduced muscle mass and strength (Visser et al., 2003). Vitamin D deficiency has been reported in excess of 80% of cancer patients (Stone et al., 2011) along with being particularly prominent in patients receiving chemotherapy (Fakih et al., 2009). Likewise, a retrospective study of advanced cancer patients reported 70% vitamin D insufficiency and an association with anorexia and fatigue (Dev et al., 2011). The observation in this study of a higher proportion of men with hypogonadism and females being deplete of vitamin D

suggests a possible link with androgen pathways. In cachectic cancer patients, vitamin D gene receptor polymorphisms have been linked as predictors of a more aggressive form of cachexia (Punzi et al., 2012).

Glucocorticoids

Glucocorticoid atrophy is specific to type II fibres. Its mechanism of action is through upregulation of protein degradation pathways, upregulation of myostatin and enhanced glutamine synthetase activity (Solomon and Bouloux, 2006, Carballo-Jane et al., 2004). Glucocorticoids inhibit physiological secretion of growth hormone and appear to reduce insulin-like growth factor (IGF)-1 activity in target organs. There are several types of cachexia associated with increased cortisol levels and muscle atrophy is a dose limiting side effect of treatment with synthetic glucocorticoids (Clarke et al., 2007). In cachectic pancreatic cancer patients, elevated serum cortisol/insulin ratios have been demonstrated compared with controls (Fearon et al., 1998).

Insulin

Insulin can stimulate protein synthesis via the phosphatidylinositol 3'-kinase (PI3K)/protein kinase B (PKB or Akt) pathway (Section 1.2.6.3.2). Insulin resistance has been demonstrated in cancer cachexia (Johns et al., 2012). Likewise, patients with uncontrolled diabetes can have marked muscle atrophy (Workeneh and Bajaj, 2013).

In human cancer patients, treatment with low dose insulin therapy as part of a combination therapy regime (with the addition of indomethacin and recombinant erythropoietin) resulted in a rise in carbohydrate intake, an increase in body fat, and a fall in serum free fatty acids. However, there was no change in lean body mass (LBM), maximum exercise capacity or spontaneous physical activity (Lundholm et al., 2007).

Growth hormone (GH)

GH has several anabolic effects and can increase LBM in patients with GH deficiency (Carroll et al., 2004). This loss of muscle mass can be reversed by GH supplementation in adult GH deficiency although without noticeable impact on strength (Hoffman et al., 2004). The mechanisms are not completely understood, but in myoblasts, insulin-like growth factor (IGF)-1, phosphorylation of Janus kinase (JAK)-2, Signal Transducer and Activator of Transcription (Stat)-5 and increased Suppressor of Cytokine Signalling-2 (SOCS-2) have all been implicated (Sadowski et al., 2001, Frost et al., 2002). It has a particularly profound action on stimulating IGF-1 in the liver, but GH can also have IGF-1 independent effects.

Insulin-like growth factors

Two insulin-like growth factors have been discovered. IGF-1 is a major anabolic factor in skeletal muscle. In mice overexpressing IGF-1, muscle mass is significantly increased compared with controls (Hayashi et al., 2004, Shavlakadze et al., 2005). IGF-2 has a developmental role and involvement with muscle

differentiation (Florini et al., 1996). It has not been investigated in cachexia and thus the rest of this paragraph will focus on IGF-1.

The main effects of IGF-1 are mediated through the PI3K/Akt pathway resulting in increased muscle protein synthesis and accelerated satellite cell proliferation and differentiation. Along with muscle, many other organs produce IGF-1 in autocrine or paracrine forms, but GH causes increased circulating IGF-1 by increased synthesis in the liver (Delafontaine et al., 2004). The effects of IGF-1 are influenced by IGF-1 binding proteins (IGFBPs). There are 6 different IGFBPs which all inhibit binding of IGF-1 to its receptor, but in addition, IGFBP-1, -3 and -5 can also enhance the action of IGF-1 (Delafontaine et al., 2004). A possible role for IGF-1 in the pathogenesis of cancer cachexia has been suggested. IGF-1 signalling was impaired in the Yoshida ascites hepatoma (AH)-130 rat model of cancer cachexia (Costelli et al., 2006). However, there was no prevention of cachexia by the administration of exogenous IGF-1 and there is a lack of conclusive evidence for a role in human cancer cachexia.

Androgens

Androgens can act on satellite cells (Sinha-Hikim et al., 2003) and IGF-1 (Lewis et al., 2002). They may also cause a state of 'anti-catabolism' via anti-glucocorticoid effects (Danhaive and Rousseau, 1988, Zhao et al., 2004). Increased androgen levels are associated with increased muscle size and strength (Bhasin et al., 1996). Recent evidence also suggests a potential role for selective androgen receptor modulators (SARMs) as therapeutic agents in cachexia (Negro-Vilar, 1999, Dobs et al., 2013). Testosterone inhibits release of TNF, IL-1, IL-6 from macrophages and stimulates

production of IL-10 (D'Agostino et al., 1999, Li et al., 1993, Malkin et al., 2004). Low testosterone levels (hypogonadism) may thus lead to an increase in pro-inflammatory cytokine production. A recent study looking at hypogonadism in male cancer patients demonstrated low levels of testosterone in the presence of high plasma IL-6 (Garcia et al., 2006). Similarly, in males with pancreatic cancer, systemic inflammation and opioid use were associated with hypogonadism (Skipworth et al., 2011). In this same study, male hypogonadism and female hyperoestrogenism were associated with shortened survival.

1.2.6.3 **End-organ changes: protein degradation and synthesis in cancer cachexia**

In healthy adults, skeletal muscle mass is maintained by physical activity and nutritional status, reflecting a balance between protein synthesis (anabolism) and degradation (catabolism). A predominance of either will result in muscle hypertrophy or atrophy. Muscle is a dynamic organ with an average daily turnover rate in human adults of 1-2% (Fearon et al., 1988). The IGF-1/PI3K/Akt/mammalian target of rapamycin (mTOR) pathway is the key regulator of anabolism in skeletal muscle. Protein synthesis begins with the association of met-tRNA and the 40S ribosome, a process regulated by eukaryotic initiation factor 2 (eIF2), which in turn is influenced by external signals integrated through mTOR. Intracellular protein catabolism/breakdown involves four major systems: the UPP, the autophagy (lysosomal) pathway, caspases, and the calcium-dependent calpain pathways. The individual prominence of each of these anabolic and catabolic pathways in muscle

wasting conditions is still unclear. In cancer cachexia, there is a general body of opinion that muscle degradation is more important than a reduction in protein synthesis (Argiles et al., 1999). However, the evidence for a predominance of a reduction in protein synthesis, an increase in protein degradation or a combination of both is less conclusive.

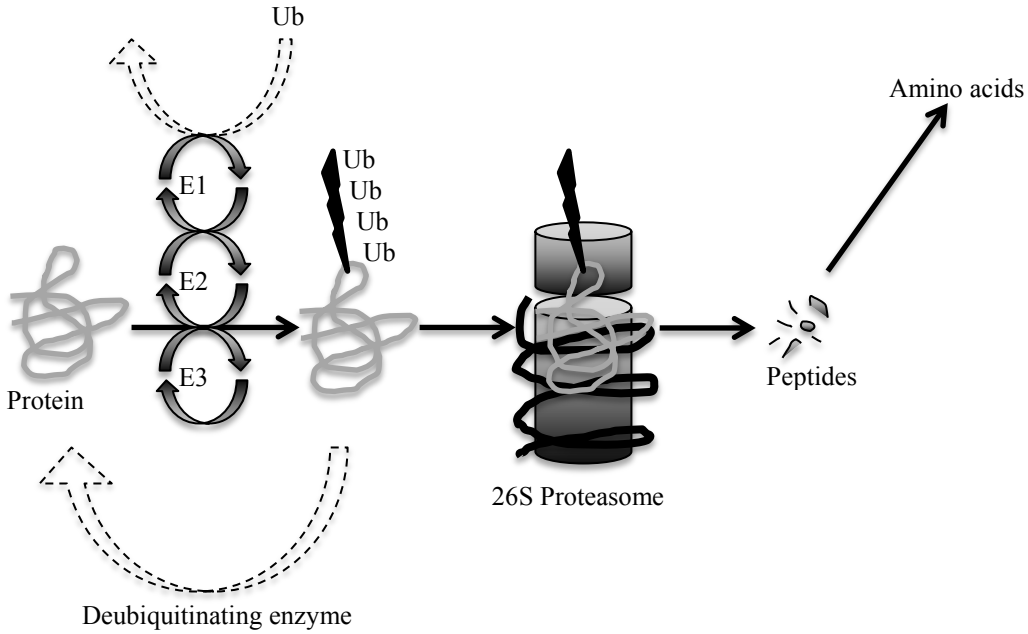
This section will discuss the key signalling pathways that are thought to be relevant in muscle wasting of cancer cachexia. It should also be noted that most molecular hypotheses relating to cancer cachexia have been generated using pre-clinical models or reflect biochemical concepts (Tisdale, 2009) and there has been little progress in relating these potential mechanisms to changes observed in patients. Due to this lack of data, much of pathway detail is derived from animal and cell experiments or other models. The first part will outline the key mechanisms of muscle protein degradation and synthesis followed by a discussion about key components of and relationships between these pathways in the context of cancer cachexia.

1.2.6.3.1 PROTEIN DEGRADATION

UBIQUITIN PROTEASOME PATHWAY

The discovery, in the 1980s, of ubiquitin and proteasomal degradation of substrate proteins culminated in the Nobel Prize in Chemistry being awarded in 2004 to Avram Hershko, Aaron Ciechanover, and Irwin Rose (Nobelprize.org, 2004). Since this discovery, there has been a wealth of data confirming UPP to be a key path for degradation of proteins in mammalian cells responsible for the bulk of proteolysis in the cytosol (Rock et al., 1994) (Figure 1.5).

FIGURE 1.5: THE UBIQUITIN PROTEASOME PATHWAY



Schematic of the ubiquitin proteasome pathway adapted from Jagoe et al (Jagoe and Goldberg, 2001). Proteins are tagged by ubiquitin via the E3 ligases before being delivered to the core proteasome machinery for degradation. This results in rapid release of peptides that are hydrolysed into amino acids in the cytosol. Abbreviations: Ub, Ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin protein ligase.

The proteasome may comprise as much as 1% of the total cell protein (Jagoe and Goldberg, 2001), is a very large complex (2000kDa) made up of at least 50 subunits, and is unlike typical proteases in that it requires ATP for protein degradation. The 26S core is comprised of a 19s 'lid' and a cylindrical 20S central proteasome. Within this central 20S core, there are three different proteolytic mechanisms that digest proteins.

Once a protein has been identified for degradation, there are three enzyme systems that are involved in covalent linkage from an internal lysine on the substrate to a chain of ubiquitin molecules:

- E1 is the ubiquitin activating enzyme
- E2 is a ubiquitin carrier protein
- E3 is a ubiquitin protein ligase

The E3 binds both the protein substrate and the E2 (carrying the activated ubiquitin), with the resultant transfer of the activated ubiquitin from the E2 to the substrate. Once there is a chain containing four or more ubiquitin molecules, the complex is degraded rapidly into small peptides (3-25 residues in length) (Jagoe and Goldberg, 2001), which are released by the proteasome and rapidly hydrolysed to amino acids in the cytosol. The poly-ubiquitin chain is also broken down to release free ubiquitin, which can then be re-used in degrading other substrates.

The E3 ligases play a pivotal role in determining which proteins pass into the proteasome. This is because each E3 ligase (functioning with a specific E2) exclusively binds individual proteins or types of proteins. Numerous E3s from

several distinct families have been discovered, and there are likely to be several hundred E3 ligases yet to be identified (Jagoe and Goldberg, 2001).

AUTOPHAGY/LYSOSOMAL PATHWAY

Autophagy is a strictly regulated lysosomal pathway responsible for the degradation of cytoplasmic material and organelles. Proteases in this system are located inside lysosomal structures and are known as cathepsins (Goll et al., 2008). It is activated during stress conditions such as amino acid starvation, unfolded protein response or viral infection. There are three different autophagic routes known that are dependent on the delivery route of the cytoplasmic material to the lysosomal lumen:

- 1) macro-autophagy (or simply, autophagy); a portion of cytoplasm to be degraded is wrapped inside a specialised organelle, the autophagosome, which then fuses with lysosomal vesicles and delivers the engulfed cytoplasm for degradation
- 2) micro-autophagy; lysosomal membrane itself sequesters a portion of cytoplasm by a process that resembles pinching off of phagosomes or pinosomes from the plasma membrane
- 3) chaperone-mediated autophagy; proteins possessing a specific sequence signal are transported from the cytoplasm, through the lysosomal membrane to the lysosomal lumen (Bonaldo and Sandri, 2013).

Autophagins, a class of cysteine proteases putatively involved in the formation of autophagosomes, are particularly abundant in the skeletal muscle (Marino et al.,

2003). Within muscle, it has not been established whether micro- and chaperone mediated autophagy play a role, and most of the data from muscle relates to the role of macro-autophagy in the autophagic process (Bonaldo and Sandri, 2013). Given this, the other two types will not be discussed any further and macro-autophagy will be referred to as autophagy from now on in the text.

The steps involved in autophagy are as follows (Klionsky et al., 2008):

1. Induction by a stress signal such as starvation,
2. The formation of an autophagosome (double membrane bound),
3. Autophagosomes receive lysosomal constituents such as lysosomal membrane proteins and proton pumps, by the fusion with endosomes or multi-vesicular bodies,
4. Finally autophagosomes fuse with lysosomes – after fusion with endosomes or lysosomes, autophagosomes are called amphisomes or autolysosomes respectively

Within these the material is then degraded and either the degradation products are transported back to the cytoplasm where they can be reused for biosynthesis or energy production, they become lysosomes that are able to undergo fusion with endosomes or autophagosomes or if the degradation of the cargo is not complete, the autolysosome becomes a residual body containing indigestible material and lipofuscin pigment.

CALPAINS

Calcium dependent proteolysis relies on the activity of cysteine proteases, which are known as calpains (calcium-activated neutral protease) – first reported by Guroff in 1964 (Guroff, 1964). There are at least 15 different calpain family members, and although they are thought to be important in tissue injury, necrosis and autolysis, the role of all members has not yet been established fully. Their activity is regulated by the relative concentration of intracellular calcium, enzyme phosphorylation and by calpastatin levels (a physiological calpain-specific inhibitor). The physiological significance (i.e. whether they function in normal turnover of any cell proteins) is unclear because they only have limited proteolytic ability. One theory is that this results in irreversible modifications of their substrates that lead to activity changes or to degradation by other proteolytic systems (Saido et al., 1994, Costelli et al., 2005, Williams et al., 1999b) – i.e. they exert a regulatory rather than a digestive function. Evidence would suggest that in skeletal muscle, they are not involved at all in the bulk degradation of sarcoplasmic or sarcolemmal proteins (Goll et al., 2003), but rather in myofibrillar degradation by disrupting thick and thin filaments from myofibrils permitting subsequent steps in which suitable protein substrates are allowed to gain access to the degradative (e.g. UPP) machinery (Goll et al., 2003, Hasselgren et al., 2002, Costelli et al., 2005). In atrophying muscle, calpains can only play a major role if cytosolic calcium homeostasis is perturbed by cell injury (Anderson et al., 1998, Tidball and Spencer, 2000).

CASPASES

The caspases are a family of cysteine-dependent aspartate-directed proteases, which are involved in apoptosis (Goll et al., 2008, Costelli et al., 2005). Apoptosis is a mechanism for ordered disruption and disposal of proteins in a damaged cell in order to maintain homeostasis and tissue integrity (MacKenzie and Clark, 2012, Dupont-Versteegden, 2006). Events such as viral infections, hormones and toxic stimuli can induce apoptosis (MacKenzie and Clark, 2012) which at the molecular level is dependent on the balance between pro- and anti-apoptotic proteins (e.g. Bcl-2 family, heat shock proteins and inhibitors of apoptosis proteins (Dupont-Versteegden, 2006). Caspases can be inflammatory cytokine activators (caspases 1 and 5) or apoptotic. This latter group is further divided into the initiators of apoptosis (caspases 2, 8, 9, 10 and 12) and the apoptotic effectors (caspases 3, 6 and 7) (Dupont-Versteegden, 2006, MacKenzie and Clark, 2012). The apoptotic pathway is well characterised and progresses through the following sequence in the dying cell:

- nuclear and cytoplasmic condensation
- plasma membrane blebbing
- release of apoptotic bodies (small membrane-enclosed particles containing cellular components)
- disposal by phagocytes or neighbouring cells which recognise the presence of apoptotic bodies (Favaloro et al., 2012)

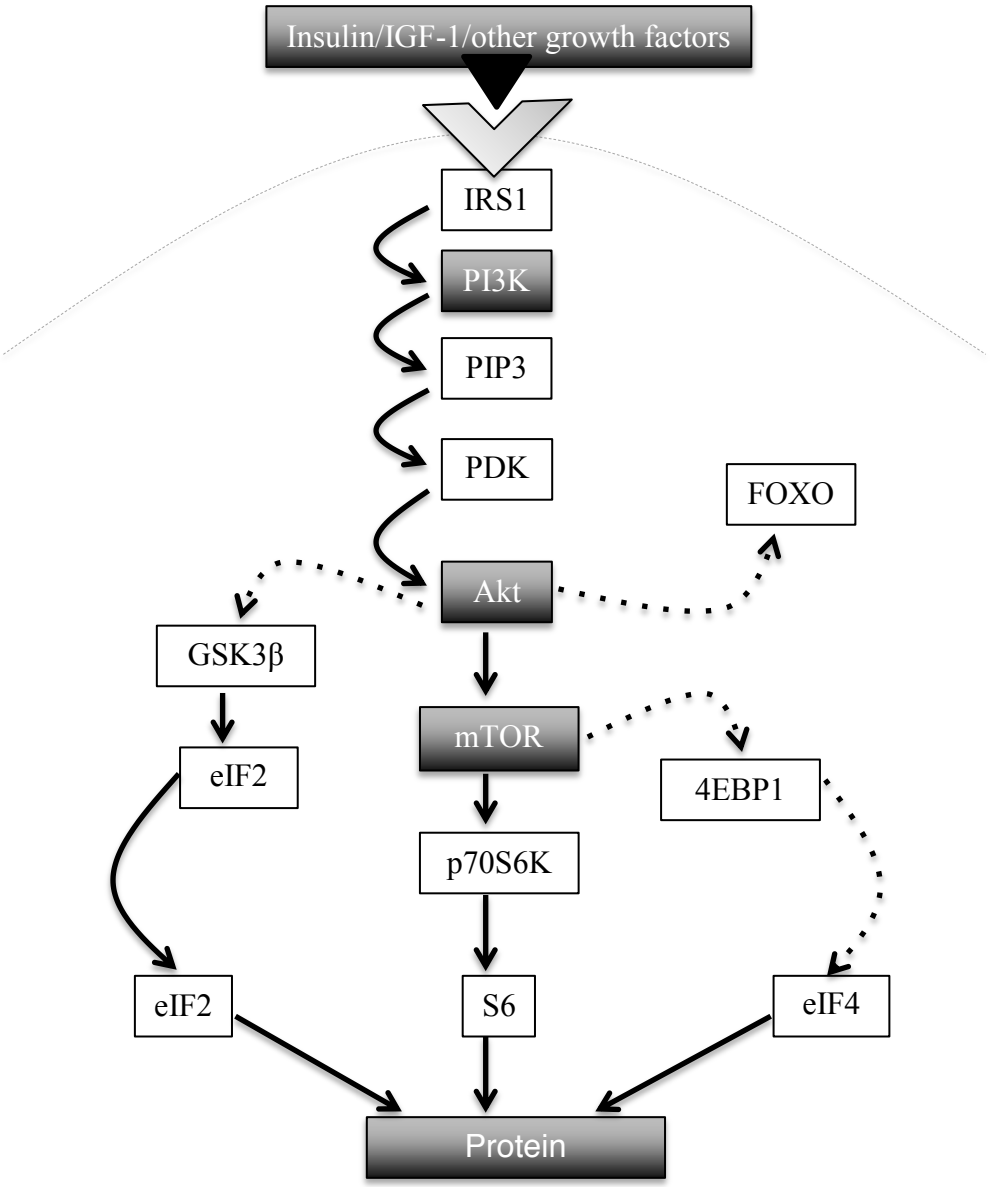
1.2.6.3.2 PROTEIN SYNTHESIS

Muscle hypertrophy is the result of a greater rate of protein synthesis (anabolism) than protein degradation (catabolism). Exercise, amino acids and various hormones are all thought to influence the rate of protein synthesis. At the cellular level, there are two main determinants: translational efficiency (protein synthesis per unit amount of RNA) and translational capacity (total ribosomal content per unit tissue) (Millward et al., 1973). The majority of research has focussed on the Akt/mTOR pathway, which is thought to be the main driver of translational efficiency, and also has a role in ribosomal biogenesis (Rennie et al., 2004). However, more recently, the β-catenin/myelocytamotosis (Myc) signalling pathway, which operates independently of the mTOR pathway, has also been implicated in the regulation of ribosomal biogenesis (translational capacity) (McCarthy and Esser, 2010).

The Akt/mTOR pathway (Figure 1.6) has been strongly implicated in skeletal muscle hypertrophy both in vitro (Rommel et al., 2001) and in vivo (Bodine et al., 2001b). Not only is there evidence that activation of the pathway and its downstream targets p70 ribosomal S6 kinase (p70S6K) and 4E binding protein (4E-BP1, also known as PHAS-1) is involved in regulating skeletal muscle fibre size, but also that pathway activation can oppose muscle atrophy induced by disease.

The Akt pathway can be activated by insulin or IGF-1 (Sandri, 2008). IGF-1 is well documented as a muscle growth-promoter and is synthesised by the liver under GH control. This produces IGF-1 both in the circulation and locally by skeletal muscle

FIGURE 1.6: THE IGF-1/PI3K/AKT/MTOR ANABOLIC PATHWAY



Schematic illustrating the main components of the IGF-1/PI3K/Akt/mTOR protein synthesis pathway. Solid arrows represent activation and dashed arrows represent inhibition. Abbreviations: IGF-1, insulin-like growth factor 1; IRS-1, insulin receptor substrate-1; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PDK1, phosphoinositide-dependent kinase-1; Akt, Ak strain thymoma/protein kinase B; FOXO, forkhead box sub-group O; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal S6 kinase; 4EBP1, 4E binding protein 1; eIF, elongation initiation factor; GSKβ, glycogen synthase kinase 3β.

(IGF-1 splicing products). This in turn activates Akt. Of the three Akt genes (Akt1/PKB-α, Akt2/PKB-β and Akt3/PKB-γ), Akt1 and 2 are expressed at higher levels in muscle, with Akt3 being predominant in the brain. Each has distinct functions evidenced by the phenotype of knockout mice: Akt1 null mice have growth retardation and muscle atrophy, Akt2 null mice suffer from Type II diabetes like syndrome and Akt3 null mice have impaired brain development (Yang et al., 2004).

When Akt activates mTOR protein synthesis is promoted through the action of its downstream targets. These are 4E-BP1, which stimulates protein translation when phosphorylated and p70S6K, which leads to the formation of the translation initiation complex (Weigl, 2012).

1.2.6.3.3 CATABOLIC/ANABOLIC PATHWAY ACTIVATION AND INTERACTION IN CANCER CACHEXIA

Given the complexity of human biology in both health and disease, it would seem inconceivable that a single pathway is responsible for the profound tissue changes and muscle wasting evident in cancer cachexia. The body may respond to the activation or depression of a degradation pathway with a compensatory change in synthetic pathways or vice versa. Several recent studies in cachexia have made a concerted effort to link pathways or search for additional novel factors implicated in muscle atrophy. This section will discuss these findings.

UBIQUITIN PROTEASOME PATHWAY/E3 UBIQUITIN LIGASES

In many models of cachexia, including cancer, the UPP is thought to be fundamental in the process of muscle atrophy (Jagoe and Goldberg, 2001). In 2001, two research groups, led by Alfred Goldberg and David Glass, independently identified two muscle-specific E3 ubiquitin ligases. Muscle RING-Finger protein-1 (MuRF1) and Muscle Atrophy F-box (MAFBx)/atrogin-1 (named differently by the two labs) were shown to be upregulated in many animal models of muscle wasting, including cancer cachexia (Bodine et al., 2001a, Gomes et al., 2001). This has been used to provide an argument for a major contribution of the UPP in muscle wasting, such that these genes are now measured as surrogate indicators of UPP activation. Similarly, microarray profiling of four models of muscle atrophy (cancer cachexia, diabetes mellitus, uraemia, fasting) demonstrated a common signature of genes termed 'atrogenes' which included MuRF1, MAFBx/atrogin-1 and components of the ubiquitin proteasome (Lecker et al., 2004). More recently, the identification of a third ubiquitin ligase, termed E3α-II, in pre-clinical models of cancer cachexia adds further evidence of the key role of the UPP in muscle atrophy (Kwak et al., 2004).

However, human investigations have failed to be as conclusive. Studies including patients in intensive care units with sepsis, following bed rest, amputation for vascular disease, limb immobilisation, COPD, amyotrophic lateral sclerosis and ageing have demonstrated both increased and decreased expression of MuRF1 and MAFBx/atrogin-1 (Fredriksson et al., 2008, Doucet et al., 2007, Salanova et al., 2008, Leger et al., 2006, de Palma et al., 2008, Edstrom et al., 2006). Likewise, investigations of UPP activity have shown similar levels to healthy controls in

patients with lung cancer and weight-loss <10% (termed pre-cachexia by the authors) (Op den Kamp et al., 2012). Another study in lung cancer patients with low weight-loss (mean 2.9%) demonstrated no change in components of the UPP using Northern blotting, but suggested that the lysosomal pathway was increased (Jagoe et al., 2002). In contrast, in gastric cancer patients with average weight-loss of 5.2%, increased UPP activity (determined by measurement of RNA and cleavage of specific fluorogenic substrates) was observed compared with controls, with a further effect with increasing tumour stage, weight-loss and lower albumin (Bossola et al., 2003). The same group also measured higher levels of ubiquitin mRNA in gastric cancer patients compared with controls, but there was no relationship with increasing weight-loss. This led the authors to conclude that the UPP was activated early in disease before there was overt clinical evidence of cachexia (Bossola et al., 2001). In another investigation using dot blot for components of the UPP in patients with various cancers and minimal weight change, mRNA levels were increased by 2-4x in cancer patients (Williams et al., 1999a). The authors similarly concluded that the UPP was upregulated before protein breakdown is increased given that patients had minimal weight-loss. The concept of changes in activation of the UPP according to where a patient is on the cancer cachexia journey is supported by the observations by Khal et al in both humans with cancer cachexia (Khal et al., 2005a) and the MAC16 murine cachexia model (Khal et al., 2005b). However, in these studies, UPP activity seemed to increase after a threshold of ~10% weight-loss with a peak between 12-19% weight-loss. Furthermore, UPP activity seemed to decrease after 20% weight-loss suggesting that other mechanisms such as a reduction in protein synthesis may be more relevant after this point.

AUTOPHAGY/LYSOSOMAL PATHWAY

The autophagy pathway is necessary to drive substrates to lysosomes, and lysosomal proteolysis is dependent on the activity of cathepsins (Attaix and Bechet, 2007). Previous muscle incubation research in muscles from cachectic animals suggested that autophagy played an insignificant role in overall protein degradation (Baracos et al., 1995, Llovera et al., 1995, Temparis et al., 1994, Costelli et al., 2005). Contrary to this, an elevation of total lysosomal protease activities has been seen in muscles and liver of tumour bearing rats (Greenbaum and Sutherland, 1983, Lundholm et al., 1980, Tessitore et al., 1994) along with increased muscle levels of cathepsin L mRNA in septic rats (Deval et al., 2001). Likewise, activation of autophagy has been demonstrated in C2C12 myotubes (Zhao et al., 2007, Mordier et al., 2000). Furthermore, in animal models of denervation and starvation induced atrophy, induction of several autophagy related genes has been demonstrated (Mammucari et al., 2007). These included light chain (LC) 3, Gamma-Aminobutyric Acid Receptor-Associated Protein-like (GABARAP)-1, Bcl-2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) and BNIP3l, all of which had been on the 'atrogene' list previously reported by Lecker et al (Lecker et al., 2004).

Autophagy has also been demonstrated to play a role in cardiac atrophy associated with cancer cachexia (Cosper and Leinwand, 2011). In the colon-26 tumour mouse model, autophagy (without UPP activation) in cardiac muscle was demonstrated to be responsible for loss of both myofibrillar and sarcomeric proteins, which is in contrast to skeletal muscle where myosin appears to be a selective target. Furthermore, male mice lost more body, muscle and cardiac mass than females and

at a faster rate. This intriguing observation requires further study, but raises questions about a potential role of sexual dimorphism in cancer cachexia.

Human studies have reported increased cathepsin D enzyme activity in cancer patients (Schersten and Lundholm, 1972) and cathepsin B mRNA in lung cancer patients (Jagoe et al., 2002). In the latter study, cathepsins B levels were higher in early versus late stage tumours. Components of the UPP were unchanged in cancer patients, and thus the authors concluded that lysosomal proteolysis may be relevant early and the UPP may be activated later in the disease process (Jagoe et al., 2002). This perhaps has some appeal because, although one study detected radio-labelled myofibrillar proteins in lysosomes (Gerard and Schneider, 1979), there is other evidence suggests that lysosomal proteolysis in isolation is not sufficient to degrade myofibrillar proteins (Lowell et al., 1986, Furuno et al., 1990, Cohen et al., 2009).

Forkhead box O (FOXO) TRANSCRIPTION FACTORS

FOXO transcription factors are a Forkhead family of proteins, which are now thought to be an important link between proteasomal and lysosomal proteolysis. In humans there are 39 distinct members and 19 subgroups (A-S). The O group is regulated by the insulin/IGF-1/PI3K/Akt signalling pathway and there are 3 members in mammalian cells: FOXO 1 (also known as FKHR), FOXO3 (FKHRL1) and FOXO4 (AFX) (Sandri et al., 2004, Carter and Brunet, 2007). FOXO is inactive in its phosphorylated state induced by Akt, where it is exported to the cytoplasm (Brunet et al., 1999). Conversely, FOXO is activated by dephosphorylation and translocation to the nucleus (Ramaswamy et al., 2002, Sandri et al., 2004). In the

context of cachexia, FOXO1 was found amongst the list of 'atrogenes' (Lecker et al., 2004), and all have been implicated in pre-clinical models of muscle atrophy.

FOXO3 activation leads to atrophy of muscle fibres (Sandri et al., 2006), and in starvation and glucocorticoid induced atrophy of myotubes and mice, FOXO transcription factors were shown to activate the E3 ligases atrogin-1 and MuRF and other 'atrogenes' (Sandri et al., 2004, Stitt et al., 2004). Overexpression of Akt could inhibit this response confirming relevance of this pathway in cachexia (Sandri et al., 2004). Subsequently, the key role of FOXO3 in induction of autophagy in skeletal muscle in vivo was also demonstrated. Mammucari et al reported upregulation of several autophagy genes by FOXO3 (Mammucari et al., 2007) including LC3, GABARAPL1, BNIP3 and BNIP3l, which comprised part of the 'atrogene' list (Lecker et al., 2004). BNIP3 was shown to be a major effector of FOXO3 mediated autophagy with its protein expression being induced by FOXO (Mammucari et al., 2007, Mammucari et al., 2008). The co-ordinate activation by FOXO transcription factors of both the proteasome and especially the autophagy pathway was illustrated in myotubes and mice. FOXO3 overexpression led to stimulation of both pathways, but with a 3 fold greater increase in lysosomal proteolysis (Zhao et al., 2007, Zhao et al., 2008). The authors suggested that under FOXO control, the UPP was responsible for degradation of myofibrillar proteins whereas activation of autophagy accounted for loss of mitochondria and endurance.

In humans, reduced levels of Akt and phosphorylated (inactive) FOXO3 have been observed in the skeletal muscle of cachectic compared with non-cachectic pancreatic

cancer patients. However, an unexplained twofold reduction in the levels of FOXO1 and FOXO3 protein was also observed making the data challenging to interpret (Schmitt et al., 2007).

CALPAINS

Calpains have also been implicated in the degradation of sarcomeric and cytoskeletal proteins in cultured myotubes (Purintrapiban et al., 2003). In this particular study, the addition of a calpain inhibitor resulted in a 20% reduction in overall protein degradation. Similarly, this was reported in a murine hindlimb suspension model where inhibition of calpain activity preserved sarcomere structure and furthermore prevented a fall in the isometric force generating capability (Salazar et al., 2010). Gastric cancer patients with minimal weight-loss (average 1.24%) had an increase in Rectus abdominis muscle calpain activity by 70% compared with controls, suggesting a role for the calpains early in the cachexia journey before significant clinical changes are apparent (Smith et al., 2011).

CASPASES

A role for caspases in muscle protein degradation was suggested due to the observations of reduced myonuclei numbers in muscle atrophy of heart failure or Duchenne muscular dystrophy (Adams et al., 2001, Sandri, 2002). Caspase-3 has been shown to cleave actomyosin complexes and caspase-3 inhibitors can prevent accumulation of actin fragments in skeletal muscle of diabetic or uremic rats suggesting that activation of caspase-3 may be an initial step in enhanced muscle

protein degradation (Du et al., 2005). However, other authors disagree with these findings suggesting that non-apoptotic cells would be unlikely to activate sufficient caspase-3 to contribute to the turnover of myofibrillar proteins (Goll et al., 2008).

There is evidence of apoptosis in skeletal muscle in the Lewis lung carcinoma mouse and AH-130 hepatoma animal models of cachexia (van Royen et al., 2000), but a role in human cancer cachexia has not been proven. In gastric cancer patients with mild-moderate weight-loss (mean 6%) there were similar numbers of apoptotic myonuclei compared with controls and no increase in caspase-1 or caspase-3 on immunohistochemistry (Bossola et al., 2006).

NFκB PATHWAY

The NFκB family are pivotal for the modulation of several cellular processes – apoptosis, differentiation, inflammation and stress responses (Hayden and Ghosh, 2004, Oeckinghaus et al., 2011, Acharyya and Guttridge, 2007). There are 5 known NFκB transcription factors, all of which are expressed in skeletal muscle with p65/p50 being the prototypical heterodimer. In the inactive state, they are bound to the inhibitor of kappa B (IκB) in the cytoplasm and translocate to the nucleus on activation. TNFα, IL-1β and PIF can all activate the NFκB pathway, and the PI3K/Akt pathway also has some regulatory activity by phosphorylation of IκB kinase (IKK), which activates NFκB. There may also be interplay between the NFκB pathway and the FOXO transcription factors with IKK negatively regulating FOXO3a independent of Akt (Hu et al., 2004). In myotubes and mice, TNF and IFN-γ induced activation of NFκB with downregulation of myoblast determination

protein (MyoD) (essential for repair of damaged tissue and regulates skeletal muscle differentiation) and dysfunction of skeletal myofibres was demonstrated (Guttridge et al., 2000). Likewise, chronic muscle specific NFκB activation in transgenic mice led to profound muscle wasting due to accelerated ubiquitin proteasome dependent proteolysis (Cai et al., 2004).

In newly diagnosed lung cancer patients with pre-cachexia (defined as weight-loss <10%), although plasma markers of systemic inflammation were increased, NFκB dependent signalling was similar to the healthy controls (Op den Kamp et al., 2012). However, in gastric cancer patients with mean weight-loss of 10.9%, there was a 25% increase in phospho-p65, and a 25% decrease in IκBα protein expression in cancer patients compared with controls (Rhoads et al., 2010). These differences did not relate to either tumour stage or cachexia suggesting that change in NFκB activity occurred early and was sustained with increasing weight-loss. These contradictory results could indicate differences in signalling mechanisms according to tumour site, but require further investigation.

PROTEIN SYNTHESIS

A primary role for suppressed protein synthesis in muscle atrophy in cancer cachexia has been proposed (Emery et al., 1984a, Rennie et al., 1983). In insulin receptor knockout mice, there is evidence that reductions in muscle mass and function are due to depression of synthesis and not an increase in degradation (O'Neill et al., 2010). With such a large body of evidence for increased protein degradation in cancer cachexia, it seems hard to completely dismiss some role of the proteolytic pathways.

However, some authors have argued that the changes in catabolic pathways are purely a secondary adaptive response (Rennie et al., 1983, Emery et al., 1984a).

A reduction of protein synthesis in cachexia may occur through either inhibition of amino acid uptake, or the suppression of RNA translational efficiency/capacity (Acharyya et al., 2004). Indeed, some tumour models show as much as a 40% decrease in total muscle RNA compared with control animals (Emery et al., 1984b, Baracos et al., 1995, Acharyya et al., 2004). The concept that skeletal muscle protein synthesis in cachexia is reduced simply due to anorexia (Tisdale, 2009) seems unlikely. In animal models where anorexia is absent, depression of protein synthesis has also been demonstrated suggesting that the protein synthetic machinery is defective (Smith and Tisdale, 1993). In the MAC-16 cancer cachexia model, catabolism and anabolism appear to occur in tandem with up to a 60% fall in protein synthesis (Beck et al., 1991). In another animal model, the APC(Min/+) mouse, a reduction in protein synthesis seemed to precede a rise in proteolysis, suggesting time dependant activation of pathways (White et al., 2011).

Branched chain amino acids are substrates for protein synthesis in skeletal muscle, but can also enhance protein synthesis by initiating signal transduction pathways that modulate translation initiation (Yoshikawa et al., 2001). Leucine seems to be most potent (Eley et al., 2007) and some authors have used this observation as a rationale to supplement leucine in the diet of cachectic patients (Tisdale, 2009).

dsRNA-dependent protein kinase

The opposing views on protein synthesis and degradation in cancer cachexia may be resolved by the discovery of the role of double stranded RNA-dependent protein kinase (PKR) in muscle atrophy (Eley and Tisdale, 2007). PKR is one of four mammalian cell eIF2α kinases that responds to distinct stress conditions that affect transcription and protein synthesis. Both PIF and angiotensin II have been implicated in activation of PKR, which subsequently can activate IKK with stimulation of NFκB activity (Eley et al., 2008). The finding of increased phosphorylation of PKR and eIF2α in myotubes and MAC-16 tumour bearing mice thus provided a potential link between a depression of protein synthesis and increased degradation in cancer cachexia (Eley and Tisdale, 2007).

Likewise, the finding that levels of phospho-PKR and phosphor-eIF2α were increased in gastro-oesophageal cancer patients compared with controls supports a role for PKR as an initiator of human cancer cachexia (Eley et al., 2008).

MYOSTATIN/ACTIIB

Myostatin was first discovered in 1997 (McPherron et al., 1997) in knockout animals with muscle hypertrophy. The potential for profound effects on muscle phenotype are evident in the mutations leading to myostatin deficient cattle (Grobet et al., 1997), whippet dogs (Mosher et al., 2007) and even a human spontaneous mutation (Schuelke et al., 2004). This has led to a growing body of research investigating the potential role in muscle wasting disorders.

Physiologically myostatin appears to be a gatekeeper maintaining satellite cells in reversible quiescence (Amthor et al., 2006). Myostatin (or growth and differentiation factor 8 (GDF-8)) is a member of the TGF-β superfamily of secreted growth factors that play an important role as a negative regulator of growth and differentiation and can also promote adipogenesis (Artaza et al., 2005, Whittemore et al., 2003). It is predominantly expressed in muscle, but is also present in the circulation (Wagner et al., 2002). Follistatin appears to be a complementary antagonist to myostatin (Amthor et al., 2004). Myostatin signalling involves interaction with the myogenic regulatory factors (MRF), inhibiting the synthesis and activity of MyoD (Amthor et al., 2004, Guttridge, 2004). Myostatin is inhibited by the anabolic actions of GH (Liu et al., 2003) and in contrast dexamethasone can increase myostatin expression (Ma et al., 2001). Exercise may decrease myostatin levels, but there are conflicting results.

Myostatin acts by binding to its receptors (activin receptor (ActR) type I, ActRIIA and ActRIIB) in muscle, which initiates a signaling cascade leading to increased expression of atrogin-1 and MuRF1 and subsequent degradation of myofibrillar proteins (Glass, 2010). Direct evidence linking muscle wasting in cancer cachexia with survival has been lacking until recently. One study in colon-26 carcinoma-bearing mice demonstrated that ActRIIB antagonism prolonged survival, prevented muscle wasting and reversed muscle loss during cancer cachexia. In addition, it was shown that ActRIIB pathway activation induced the ubiquitin ligases and enhanced ubiquitination of muscle proteins. Furthermore, ActRIIB antagonism abolished

activation of the UPP and dramatically stimulated satellite cell proliferation, which presumably contributed to the rapid reversal of muscle loss seen in the treated animals (Zhou et al., 2010).

In a recent study of myostatin expression in muscle biopsies from weight stable lung and gastric cancer patients, there was evidence of an increase only amongst the gastric cancer patients (Aversa et al., 2012). This suggests that in human cancer cachexia, myostatin alterations vary by tumour site but may play a role early in the cachexia journey.

TNF receptor-associated factor (TRAF) 6

TRAFs are proteins involved in activation of various aspects of intracellular signalling(Paul et al., 2010, Paul and Kumar, 2011). TRAF6 is an important E3 ubiquitin ligase, which targets proteins for degradation (Pickart, 2001, Mukhopadhyay and Riezman, 2007). Several studies have shown TRAF6 to be related to activation of NFκB, mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways (Yang et al., 2009, Yamashita et al., 2008). TRAF6 expression level and auto-ubiquitination were enhanced in skeletal muscle during atrophy in mice (Paul et al., 2010). This study also demonstrated that through the activation of Jun N-terminal kinase (JNK)1/ 2, p38 MAPK, adenosine monophosphate-activated protein kinase (AMPK), and NFκB, skeletal muscle-restricted depletion of TRAF6 rescues myofibril degradation and preserves muscle fibre size and strength upon denervation (Paul et al., 2010). Inhibition of TRAF6 also preserves the orderly pattern of intermyofibrillar and subsarcolemmal mitochondria in denervated muscle.

These results suggest that depletion of TRAF6 prevents cancer cachexia in an experimental mouse model and provides a novel therapeutic target for prevention of skeletal muscle wasting in cancer cachexia.

Signal transducer and activation of transcription (STAT)3

Recent work has focused on STAT3 activation in skeletal muscle and has linked it with muscle wasting and the acute phase response in cancer cachexia (Bonetto et al., 2011). This contemporary study characterized serum cytokines and the muscle transcriptome in the colon-26 adenocarcinoma model of cancer cachexia. There was evidence of STAT3 activation, target gene expression and the acute phase response in both the liver and skeletal muscle of the mice. In addition, it was shown that transcription of suppressor of cytokine signalling (SOCS)3, a classical feedback inhibitor of STAT3 activation was induced. SOCS3 binds to activated Janus kinase (JAK) and inhibits STAT3 activation (Glass, 2005). However, in contrast to the high SOCS3 mRNA levels in muscle, little or no increase in SOCS3 protein either in muscle or in liver was observed. This lack of SOCS3 protein explains, in part, how high pSTAT3 levels could persist regardless of high SOCS3 RNA levels and how sustained STAT3 activation might continue to drive muscle wasting while activating its inhibitor.

DOWNSTERAM TARGETS: ALTERATIONS IN MUSCLE STRUCTURE

Myofibrillar proteins

It is presumed that the main downstream targets of cachexia are the myofibrillar proteins because of their overall abundance and functional relevance in skeletal muscle architecture (Clark et al., 2002). Normally proteins comprising the myofibril are amongst the most stable in the body, but during atrophy induced by unweighting, the myofibrillar apparatus decreases in mass to a greater extent than soluble sarcoplasmic components (Munoz et al., 1993) and results in decreased strength. As discussed earlier in the introduction, (Section 1.2.6.1) the myofibril is a precisely aligned filament system arranged in repeating units of sarcomeres with myosin being the main component of thick filaments and actin the main component of thin filaments (Seeley, 1995). The E3 ligase MuRF1 has been demonstrated to play a key role in myofibrillar degradation. It has two substrates; cardiac troponin-I (Kedar et al., 2004), and MyHC (Clarke et al., 2007). In the latter study, whilst there was selective depletion of MyHC, actin levels remained unperturbed. This selective targeting of MyHC had previously been demonstrated in both myotubes and colon-26 cachectic mice (Acharyya et al., 2004) with no alteration in levels of other myofibrillar proteins (tropomyosin, troponin, sarcomeric actin, actinin, myosin light chain (MyLC)).

However, more recent studies suggest that different mechanisms are responsible for degradation of the thick and thin filaments, and there also seems to be time dependent activation. In denervation murine models of atrophy, MuRF1 was induced after 3 days but did not cause a reduction in MyHC till 14 days after

induction (Cohen et al., 2009). There was loss of other components of the myofibril (MyLCs and myosin binding protein-C (MyBP-C), which are proteins that stabilise the thick filament) due to MuRF1 but only after 10 days. This suggested that other mechanisms were responsible for the fall in muscle mass during the first week. Similarly, given that MuRF1 did not degrade the thin filaments, the authors concluded that a MuRF1 independent mechanism must be responsible (Cohen et al., 2009). Interestingly, a study published in 2012 suggested that MyHC was not selectively targeted in cancer cachexia but rather decreased in parallel with other myofibrillar proteins (Cosper and Leinwand, 2012). Furthermore, the authors suggested previous studies had flawed muscle lysis methodology leading to the misinterpretation of selective MyHC loss. However, other research groups do not seem to have commented on experiencing similar methodological difficulties.

In humans, loss of MyHC has also been reported in patients with gastro-oesophageal cancer cachexia (Eley et al., 2008, Acharyya et al., 2005). Another study in pancreatic cancer patients with cachexia showed a 45% decrease in MyHC protein levels compared with controls (Schmitt et al., 2007).

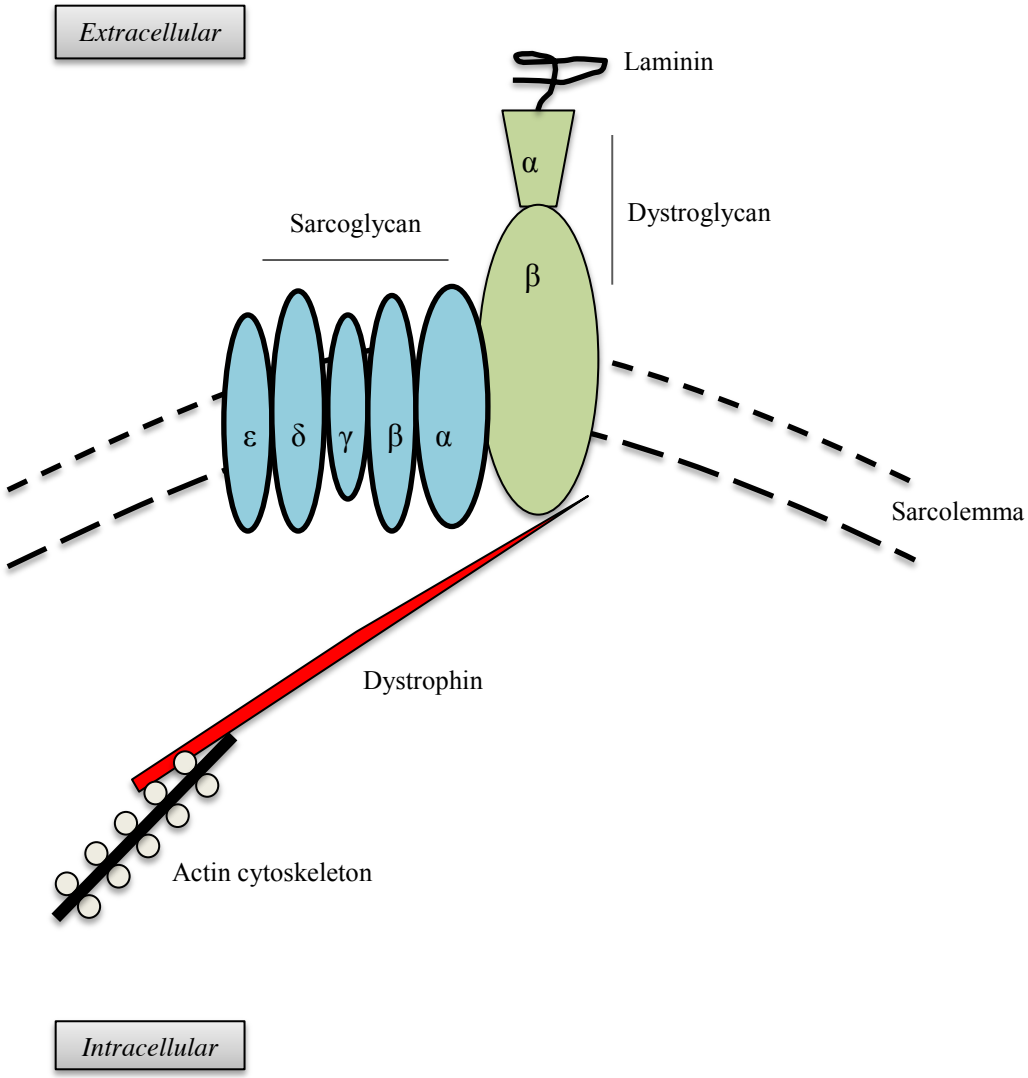
Dystrophin glycoprotein complex (DGC)

During contraction of muscle, the contractile machinery inside the myofibres must remain intimately connected with the membrane and extracellular matrix. If this does not occur, movement would be improperly transmitted and myocytes would risk damage to their membranes (Lapidos et al., 2004). One function of the DGC is just that; to provide a strong mechanical link from intracellular cytoskeleton (namely

subsarcolemmal actin) to the extracellular matrix (Figure 1.7). The DGC is a large, multimeric complex comprising a group of transmembrane, peripheral membrane, and extracellular and cytosolic proteins – mutations of which may give rise to distinct muscular dystrophies (Batchelor and Winder, 2006). It holds not just structural but also signal transduction/reception properties and is comprised of transmembrane, cytoplasmic and extracellular proteins (Batchelor and Winder, 2006, Lapidos et al., 2004). An active signal transduction pathway is maintained by interaction of the DGC with Growth factor receptor-bound protein (Grb)2 and neuronal Nitric Oxide Synthase (nNOS) (Rando, 2001, Acharyya et al., 2005).

The integral function that the DGC plays in maintaining muscle structure led to an investigation of the link between muscular dystrophy and cancer cachexia (Acharyya et al., 2005). Using the colon-26 mouse model, it was evident that the membranes of cachectic muscles were wrinkled indicating that the sarcolemma and associated basal lamina was abnormal. Confirmation of membrane damage was seen by an increase of Evans blue dye uptake in cachectic muscle. Assessment of the different components of the DGC revealed what the authors referred to as deregulated DGC (reduction of dystrophin, hyperglycosylation of β-dystroglycan and β-sarcoglycan in cachectic mouse muscle. Furthermore, deregulated DGC was also seen in 59% (16/27) of patients with gastro-oesophageal cancer but not any of the controls, and was related to poorer survival (Acharyya et al., 2005).

FIGURE 1.7: THE DYSTROPHIN GLYCOPROTEIN COMPLEX



Adapted from Acharyya (Acharyya and Guttridge, 2007). Schematic illustrating the components of the dystrophin glycoprotein complex, which provides a strong mechanical link between intracellular actin and extracellular laminin. Dystroglycan and sarcoglycan components are shown in green and blue respectively.

This part of the introduction has discussed current knowledge relating to the phenotype of cancer cachexia, along with the potential molecular mechanisms involved. The next section will provide a background on biomarkers and their relevance to disease and cachexia.

1.3 ***Biomarkers – an overview***

"He replied, when evening comes you say, it will be fair weather, for the sky is red, and in the morning, today it will be stormy, for the sky is red and overcast"
(Matthew 16v2-3a, Holy Bible, Hodder & Stoughton)

Since biblical times, red sky has been used as a predictive biomarker of weather conditions. However, although 'biomarkers' have been in everyday use for many years, the explicit terminology only became more widely used in the scientific literature during the 1960s. Table 1.5 outlines some of the historical landmarks in biomarker discovery with some examples.

Traditionally, biomarkers tended to be physiological variables (e.g. pyrexia as a biomarker of infection, a heart murmur can be seen as a biomarker of cardiac valve disease, or blood pressure as a biomarker of hypertension). Over the past century, the increasing use of biochemistry in healthcare has led to markers such as blood glucose in diabetes and creatinine in renal disease. In recent decades, the field of biomarkers has expanded exponentially with the advent of more sophisticated and complex techniques and technologies (such as microarrays or deep nucleotide sequencing) and the mapping of the Human Genome leading to discovery of many

Table 1.5: Historical landmarks in the discovery and development of biomarkers

Year	Landmark
1847	Urinary Bence Jones protein was used as a cancer biomarker
1954	Test developed for measuring transaminases in myocardial infarction
1967	Serum creatine phosphokinase used as an improved biomarker test for myocardial infarction
1971	Carcinoembryonic antigen reported as a biomarker of cancer
1987	Troponin I reported as a sensitive biomarker for myocardial infarction
2000	Potential to discover gene biomarkers with the completion of sequencing the human genome

Adapted from The Handbook of Biomarkers (Jain, 2010)

molecular biomarkers of disease (Kurian et al., 2007). In parallel with this, the pharmaceutical industries have become increasingly interested in the potential for biomarkers to be used as measures of drug response, potential drug targets and surrogate markers of endpoints allowing more rapid assessment of treatment effects during trials.

The increasing availability of molecular biomarkers has revolutionised everyday clinical medical practice. There has been increasing interest and dependency amongst physicians on biomarkers used to diagnose conditions (e.g. troponin in myocardial ischaemia/infarction, imaging modalities – Positron Emission Tomography (PET)-CT), monitor response to treatments (e.g. carcinoembryonic antigen (CEA), antibiotic levels) along with screening to predict development of disease or at risk populations (e.g. cervical smears, prostate specific antigen (PSA), genetic mutations). The drive towards personalised medicine is closely linked to biomarker discovery and has generated a wealth of data allowing prediction of an individual patient's likely response to treatment or development of side effects. Furthermore, it is now possible to fill online tools with levels of markers (cholesterol, height/weight) and risk factors (lack of exercise, diet) to give a prediction of your personal risk of developing a number of diseases (cardiovascular/stroke/cancer) (Siteman Cancer Center).

It has been estimated that the global biomarker market will increase to £12.7 billion by 2014 (Henry, 2011). In late 2006 the biomarkers consortium was established as a unique partnership between the Food and Drug Administration (FDA), National

Institutes of Health (NIH) and industry in an effort to co-ordinate and facilitate biomarker research. Indeed, this consortium now funds a range of projects ranging from plasma biomarkers of Alzheimer's disease through to diagnostic markers of ageing (Consortium). It is thus apparent that the field of biomarker research and application to everyday practice is going to continue to expand.

1.3.1 **Definition of a biomarker**

Many different definitions of biomarkers have been proposed, mainly associated with the specific context of their use. In the broadest sense, a biomarker is a measurable entity that indicates the presence or absence of a condition. There is a temptation to used the terms 'biomarker' and 'surrogate' interchangeably. However, there is a clear hierarchical distinction between biomarkers and surrogate endpoints (Jain, 2010). This was highlighted in 1998, when the Biomarker Definitions Working Group (part of the NIH) presented (and subsequently published in 2001) their preferred definitions for biomarkers and surrogate endpoints:

Biomarker - A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Clinical Endpoint - A characteristic or variable that reflects how a patient feels, functions or survives.

Surrogate Endpoint - a biomarker intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm, or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence.

Biomarker Definitions Working Group - 1998

(Biomarkers Definitions Working, 2001)

1.3.2 **Categories and uses of biomarkers**

Biomarkers can be categorised in many ways such as by method (e.g. imaging vs. molecular) or tissue compartment (e.g. blood versus muscle versus urine). However, more helpful is the proposal by Ransohoff in 2003 of two major categories of approaches presently available for the discovery of new biomarkers (Ransohoff, 2003):

- hypothesis-driven research: investigation of molecules believed to be involved in cancer biology, putative candidate genes or proteins are considered one by one as possible biomarkers
- discovery-based research: gene-expression patterns or mass-spectromic peaks which may be used to select and identify new candidate genes or proteins by conventional methods or be used themselves as patterns or signatures of disease (Gion and Daidone, 2004)

There are almost an infinite number of varied potential uses for biomarkers both as research tools and in the clinical care of patients. This ranges from red hair and fair skin as a biomarker of susceptibility to skin cancer (Mitra et al., 2012), single-

nucleotide polymorphisms as biomarkers of oesophageal/pancreatic/hepatocellular cancer risk (He et al., 2013) through to monitoring of drug levels in patients (Wong et al., 2013).

Molecular and genetic based biomarkers are divided into three types with the suggestion that there should be a stepwise progression of biomarkers from 0-2 (Mildvan et al., 1997).

Type 0: NATURAL HISTORY MARKER

These can be used to identify a population predisposed to a disease, to identify patients with a disease, for staging, to predict prognosis/disease progression or to identify recurrent disease (Jain, 2010).

Type 1: BIOLOGICAL ACTIVITY MARKER

These can be used for target discovery, □valuation of drug activity, understanding mechanisms of action, toxicity and safety evaluation and investigation of pharmacodynamics/kinetics (Sahu, 2011).

Type 2: SURROGATE MARKER OF THERAPEUTIC EFFICACY

This is the use of biomarkers as surrogate endpoints.

1.3.3 **Biomarker validation – from discovery to clinical use**

Discovering a potential biomarker does not necessarily translate into it being a useful biomarker. Table 1.6 outlines the phases of biomarker development. The literature

Table 1.6: Phases of biomarker development

Phase	Description	Number of analytes	Number of samples
Discovery	Identify candidate biomarkers	1000s	10s
Qualification	Confirm differential abundance of candidates	30-100	10s
Verification	Begin to assess specificity of candidates	10s	100s
Validation and assay development	Establish sensitivity/specificity and optimise assay	4-10	Many 1000s

Adapted from Rifai et al (Rifai et al., 2006). Number of analytes = proteins expected to be evaluated as candidate biomarkers in each phase of development. Number of samples = sample requirements for each phase

is packed with descriptions of 1000s of potential biomarkers and yet relatively few have been adopted in routine clinical practice. The focus of this section is on the processes that a biomarker passes through from first discovery to the point of use.

The steps of biomarker validation have been emphasised by Pepe et al (Pepe et al., 2001), who describes five phases:

- pre-clinical exploratory phase that identifies promising directions
- clinical validation in which an assay can detect and characterise a disease
- retrospective longitudinal validation (i.e. a biomarker can detect disease at an early stage before it becomes clinically detectable or has other predictive value)
- prospective validation of the biomarker accuracy
- testing its usefulness in clinical applications to predict clinically relevant parameters (Tahara et al., 2009, Rifai et al., 2006).

Even after validation, there will be various FDA/regulatory testing and approvals and marketing hurdles to overcome (Jain, 2010). There are many reasons why a biomarker (even a valid one) may not make it into routine use. This will be discussed further in Section 1.3.6.

1.3.4 **Potential sources of biomarkers in humans**

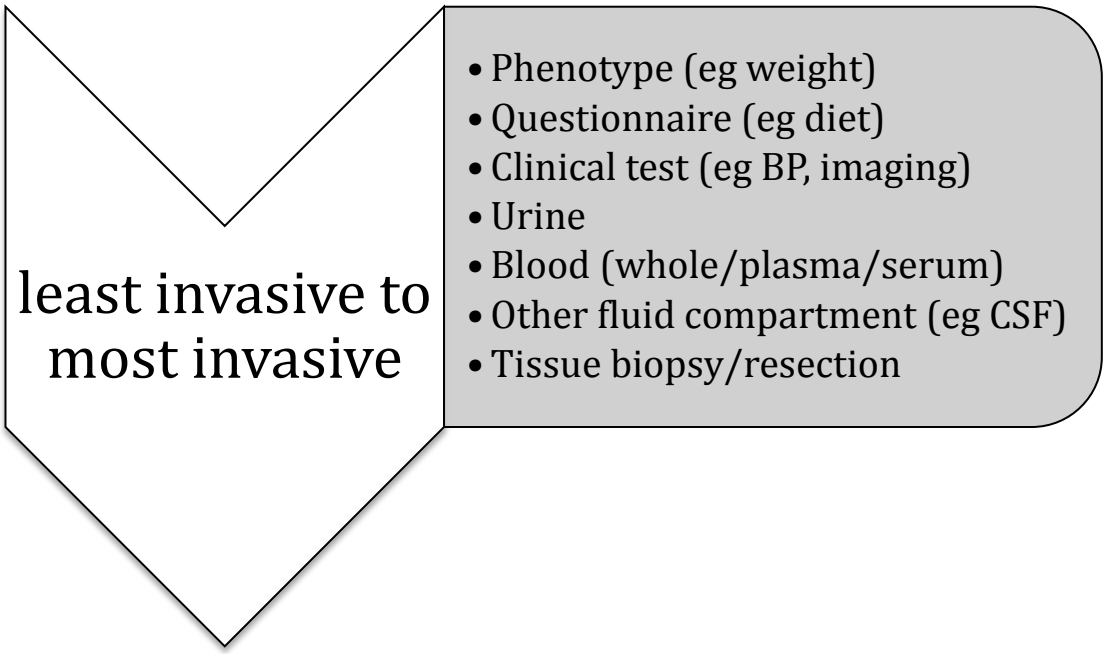
Ideally, a biomarker should be assessed with the minimum of discomfort or complexity to the patient. At the level of biomarker discovery and biomarkers which can inform pathophysiology or drug targets, more invasive methods (e.g. biopsies)

may be required until a less invasive correlate can be identified. Biomarkers are present in all parts of the human body. Figure 1.8 illustrates potential sources of biomarkers in humans ordered by degree of invasiveness.

1.3.5 **The 'ideal' biomarker**

To be clinically acceptable, the ideal biomarker should have a sensitivity and specificity close to 100%. Sensitivity refers to the percentage of positive samples identified as true positive and specificity is the percentage of negative samples that are true negative. These are important factors to consider in performing biomarker studies because higher numbers of false positives will increase the amount of work generated whereas false positives will increase the amount of cost of a study (Mayeux, 2004). In clinical practice, it is perhaps surprising to find that several of the commonly used biomarkers have relatively low values. For example PSA, used as a biomarker of prostate cancer, has a sensitivity of 86% and a specificity of 33% (positive predictive value of 41%). Despite this, the FDA has approved its use in combination with a digital rectal examination (Issaq et al., 2011, NCI, 2009). Equally other biomarkers for breast, bladder and ovarian cancer have relatively low sensitivity or specificity results (Issaq et al., 2011). These results are indicative of the complexity and heterogeneity of human beings, and it is unlikely that a test with 100% sensitivity and specificity will ever be developed. In bladder cancer, it has been shown that using multiple biomarkers gives better sensitivity and specificity (Horstmann et al., 2009) and thus using biomarker patterns, combinations or signatures may prove to be more relevant.

FIGURE 1.8: POTENTIAL SOURCES OF BIOMARKERS IN PATIENTS



Abbreviations: BP, Blood pressure; CSF, cerebrospinal fluid.

Jain has suggested that a biomarker is valid if:

> "1. It can be measured in a test system with well-established performance characteristics.
> 2. Evidence for its clinical significance has been established" (Jain, 2010).

It is also important that a biomarker is able to pick up something that is not evident with a careful clinical assessment and that the method of obtaining the biomarker is the least invasive possible, without prohibitive cost and tolerated well by patients.

1.3.6 **Problems and limitations of biomarkers**

Despite the diversity and wealth of literature describing potential biomarkers and their applications, relatively few have progressed to the point of clinical use. This can be due to inadequate sensitivity and specificity as discussed above, or financial constraints in the widespread use of the biomarker. However, there are particular idiosyncrasies to biomarker research that confound initially exciting results. Some of the potential sources of errors and problems in biomarker research are outlined below.

RECRUITMENT

In designing a biomarker study, it is important to ensure that a population is not too niche where limitations of age, gender or ethnicity can mean that the results do not apply to other populations. This is particularly relevant in genetic studies where there can be geographical variation (Beaumont, 2004). Other variables known to have an impact on the proposed biomarker should also be taken into consideration at the beginning of any investigation. For example, will tumour stage or type impact on

a proposed biomarker (Issaq et al., 2011, Horstmann et al., 2009)? Likewise, the control population should be carefully screened for any confounding co-morbidities.

METHODS

There are reported differences in the presence of certain proteins in serum versus plasma (Issaq et al., 2011) and thus explicit methods need to be defined and adhered to in order to avoid introducing error. There can also be limitations of the modality used to assess the biomarker. For example, cross-sectional imaging can be misinterpreted depending on the expertise of the radiologist, and every laboratory technique carries an error margin. Storage of tissue samples needs to be consistent and any freeze thaw cycle taken into consideration given the potential for protein/RNA changes giving false results or resulting in degradation of potential biomarkers. The statistical analysis can also lead to errors. Although many biological phenomena occur in a continuous fashion, many biomarker studies oversimplify results into positive/negative cut-offs (Gion and Daidone, 2004). Whilst this can be informative and is appealing for practical reasons, some statisticians have warned about pitfalls in using this method without first exploring correlative relationships (Altman et al., 1994, Gion and Daidone, 2004).

APPLICABILITY

Another limiting step in biomarker research is the potential for wider use. The source of the biomarker is important – carrying out a brain biopsy may be possible in a central tertiary care facility with availability of specialists, but may not be available outside this sphere. Likewise, if the method in which a proposed biomarker is

obtained is too invasive, carries significant risk, or is too costly then clinicians and patients are unlikely to embrace its use. Variations within and between equipment and the settings used will also need to be accounted for – i.e. comparison of images from an older MR scanner with lower resolution values in a district hospital with the latest specification MR scanner at the regional centre.

1.4 ***Current biomarker research in cancer cachexia***

There are relatively few papers relating to biomarkers in cancer cachexia in the literature. A search of PubMed using the terms 'biomarkers', 'cancer' and 'cachexia' identified 143 papers. Of these, 12 were foreign language and around 26 were review articles. The remaining 105 were a mixture of original investigations, case reports, treatment trials, and papers looking at biomarkers in cancer per se rather than relating to cachexia. Indeed, less than half of the papers mentioned cachexia, malnutrition, muscle wasting, or weight-loss in the title and there were few human studies. The lack of a standard definition of cachexia in the past has also introduced a unique challenge in biomarker research. If, for example, weight-loss cut-offs are used, there is an assumption that weight-loss is a robust biomarker of cachexia. The investigator is thus left with the intriguing problem of relating a biomarker to another biomarker that may or may not relate accurately to the presence of the original condition (cachexia) being examined. With the recent strive towards a unified and robust definition of cancer cachexia the hope would be that this problem would become redundant.

The majority of human cancer cachexia biomarker research is still very much focussed on the identification of potential biomarkers. Most of these have been hypothesis-driven (i.e. analysing selected candidates) and have been discussed previously in the molecular mechanisms of cachexia, Section 1.2.6. However, a couple of the more recent discovery-based research studies of biomarkers in human cancer cachexia deserve particular mention.

Blood biomarkers

Identification of serum proteins involved in pancreatic cancer cachexia (Felix et al., 2011)

This study examined serum from patients with pancreatic cancer (stage II) undergoing resectional surgery. Serum samples were analysed by mass spectrometry (CM-10 and Cu-IMAC platforms) and results of controls (n=20) and patients with (n=23) or without cachexia (n=10) were compared. 47 significant peak differences were identified of which 15 were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Four potential biomarkers (1.4-2.3x fold change) of cancer cachexia were isolated: glucagon-like peptide 1 (GLP-1), apolipoprotein (Apo)-CII and -CIII and ZAG. The novel findings of this study need to be placed into context. Cachectic patients were defined as those with a weight-loss >12% in 6 months compared with 0% in the non-cachectic group. The rationale for this is not clear from the paper and whilst the authors conclude that identified proteins could be used "for early diagnosis of cachectic pancreatic cancer patients", this has to be questioned given the severity of weight-loss where patients can hardly be classified as 'early' in the cachexia journey. Furthermore, in validating ZAG

protein levels using enzyme-linked immunosorbent assay (ELISA), there was no difference between cachectic patients and a separate group of cancer patients with weight-loss 4.5% in 6 months. This lack of additive effect with increasing weight-loss suggests that the markers may relate more closely to cancer per se or represent general illness markers. Another confounding factor was that the mean age of controls was some 20 years younger than the cachectic patients and yet there did not appear to be any attempt to control for this, nor for gender in the data analysis. In spite of this, the results confirm the potential for investigating blood in the search for biologically relevant biomarkers of cancer cachexia.

Urinary biomarkers

Mass spectromic detection of candidate protein biomarkers of cancer cachexia in human urine (Skipworth et al., 2010b)

Mass spectrometry was utilised to examine urine from control, cachectic (weight-loss >10%) and weight stable gastro-oesophageal cancer patients (n=8 each group). Higher numbers of protein species were evident in cachectic patient urine. Proteins that were prevalent in cachectic patients were associated with muscle (myosin species), cytoskeleton (α-spectrin, nischarin) and microtubules (microtubule-actin crosslinking factor/microtubule-associate protein 1B/bullous pemphigoid antigen 1). Of particular note was the presence of MyHC7/cardiac muscle/β variant and unconventional myosin species 5c, 7a, 9a and 10 in almost 40% (n=3/8) of cachectic patients whilst it was absent in all weight-stable and control patients. Although the number of patients in this study is fairly modest, the results appear to suggest a role for myosin loss in human cancer cachexia; similar to the evidence from animal

models of muscle wasting where the myofibrillar proteins are selectively targeted and membrane permeability is increased (Acharyya et al., 2005). The authors indicate that the study was "a preliminary attempt to identify urinary proteins that might be used in future biomarker studies" and indeed, the data pave the way for larger validation studies to be done.

Summary of Introduction

This introductory section of the thesis has provided an overview of current knowledge relating to the cachexia phenotype, the molecular mechanisms relevant in muscle wasting and the potential of biomarkers. Data from animal models of muscle wasting have been fundamental in improving our knowledge of pathways relevant in muscle anabolism and catabolism along with identification of potential therapeutic targets, but these mechanisms may not relate to changes observed in the human patient. For example, glucocorticoids cause profound proteolysis in pre-clinical models of cachexia (Schakman et al., 2009). In stark contrast in patients with Cushing's syndrome (characterised by elevated glucocorticoids), there was no evidence of upregulation of calpains, cathepsin D nor components of the UPP (Ralliere et al., 1997). This indicates that the UPP may not be fundamental in the more chronic wasting seen in patients with Cushing's syndrome (Williams et al., 1999a). Whether the same is true in cancer cachexia patients remains unclear. The limited number of primary human cancer cachexia biomarker investigations highlights a recurrent problem – there is limited understanding of the molecular processes that lead to cancer cachexia in humans. Human research remains at the stage of searching for biomarkers that can inform pathophysiology and/or drug

targets. Therefore, with muscle being a fundamental target in cancer cachexia, it would seem there is a pressing need for more primary investigations using muscle biopsies for biomarker discovery research before looking for correlates in blood or urine.

With this background in mind, the next section will describe the objectives of this thesis.

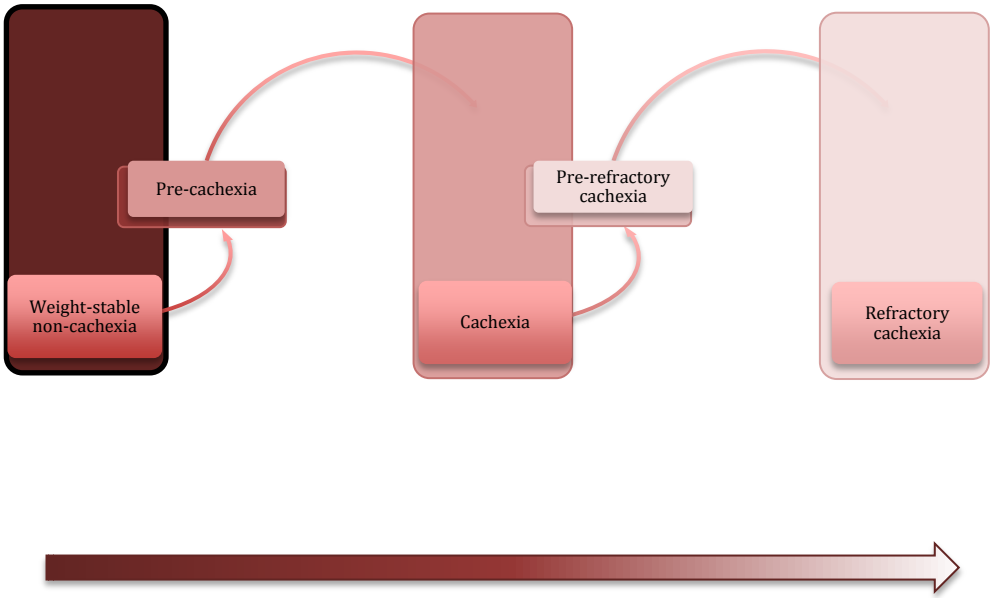
1.5 **Thesis objectives**

1.5.1 **Definition and classification of cancer cachexia**

The definitions of cachexia were discussed at the beginning of the introduction in Section 1.2.1. Despite this recent development, studies that have attempted to validate these definitions are only just coming into publication. It is thus difficult to know what would be the best definition to use in this thesis. A percentage weight-loss cut-off definition is not ideal as there will be overlap between cachectic patients and those who are pre-cachectic. Some patients in the weight stable group will go on to develop cachexia and yet results will be analysed in the 'non-cachexia' group, and thus may dampen any significant differences (Figure 1.9).

However, within everyday clinical practice, a simple above or below cut off would be more easily applicable. Likewise, more complex definitions which rely on sophisticated body composition measures using CT/MR imaging/DEXA, biochemical variables or functional parameters may limit their applicability outside

FIGURE 1.9: PHASES OF CACHEXIA



Cachexia is a journey rather than an event. Categorising patients by strict weight-loss cut-offs will inevitably include patients at the verge of losing more weight and progressing down the cachexia spectrum.

of research studies due to access to the appropriate equipment or availability of finance.

The applicability of a cross-sectional based diagnostic criteria in the context of a process which progresses with time could also be questioned. Without the availability of pre-morbid body compositional analysis, CT can only be based on population-based cut-offs that may not be so easily applicable on a patient-by-patient basis. Patients are generally able to give an estimate of pre-morbid body weight. Whilst this does rely on patient recollection and is thus open to a degree of error, there is evidence that self-reported weight is reliable (Stunkard and Albaum, 1981, Perry et al., 1995).

Therefore, this thesis is based around the definition and classification of cancer cachexia as proposed by Fearon et al in 2011 (Fearon et al., 2011). This is because it is specific to cancer cachexia, is based on an international consensus rather than from a single centre's experience, encompasses the notion that loss of skeletal muscle will have more profound impact on a patient than loss of other tissue compartments and highlights the importance of cachexia as a journey. The model that is used are patients with UGI cancer undergoing resectional surgery due to the higher percentage of patients that are cachectic according to previously reported data (Dewys et al., 1980) and the access to tissue biopsies whilst the patient is anaesthetised.

Given the profound impact skeletal muscle loss may have on patients' muscle function and QoL, the framework for this thesis is centred on loss of muscle. The

first part of the thesis will focus on staging of cancer cachexia by investigating potential biomarkers relating to early cachexia. In the second part of the thesis, the phenotype of the patient with cancer cachexia is explored by assessing muscle mass, function and quality in patients with moderate cachexia.

1.5.2 **Overall hypotheses of thesis**

1. That there are potential biomarkers of cancer cachexia in human skeletal muscle.

2. That more specific physiological phenotyping of cancer cachexia from the evaluation of skeletal muscle mass, function and ultrastructure may prove feasible, informative and be used in longitudinal studies for further biomarker discovery and validation.

1.5.3 **Aims of thesis**

A) Cachexia biomarker discovery in human skeletal muscle

1. To determine if, within skeletal muscle, changes in specific proteins or mRNA species (identified from preclinical models reported in the literature) correlate with conventional markers of cachexia in humans and might be candidate biomarkers for cancer cachexia (Chapter 3).

2. To determine from within human skeletal muscle obtained at cancer diagnosis if a systems biology approach (transcriptomics) can provide early cachexia candidate biomarkers (Chapter 4).

3. To determine if candidate biomarkers within the human skeletal muscle transcriptome can be identified by a longitudinal analysis of patients recovering from cachexia (Chapter 5).

B) Assessment of skeletal muscle mass, function and ultrastructure

1. To determine changes in skeletal muscle mass and non-contractile elements content in vivo using cross-sectional imaging and k-means statistical analysis in patients with cancer cachexia (Chapter 6).

2. To characterise the relationship between skeletal muscle mass/function (gross mechanical quality) and QoL in patients with cancer cachexia (Chapter 7).

3. To determine the nature of change in skeletal muscle at the ultrastructural level associated with cancer cachexia with a specific focus on lipid droplets (Chapter 8)

4. To determine the feasibility of a longitudinal study in patients with cancer cachexia and to assess the influence of cachexia on recovery from resectional surgery (Chapter 9).

Chapter 2: **Methods and materials**

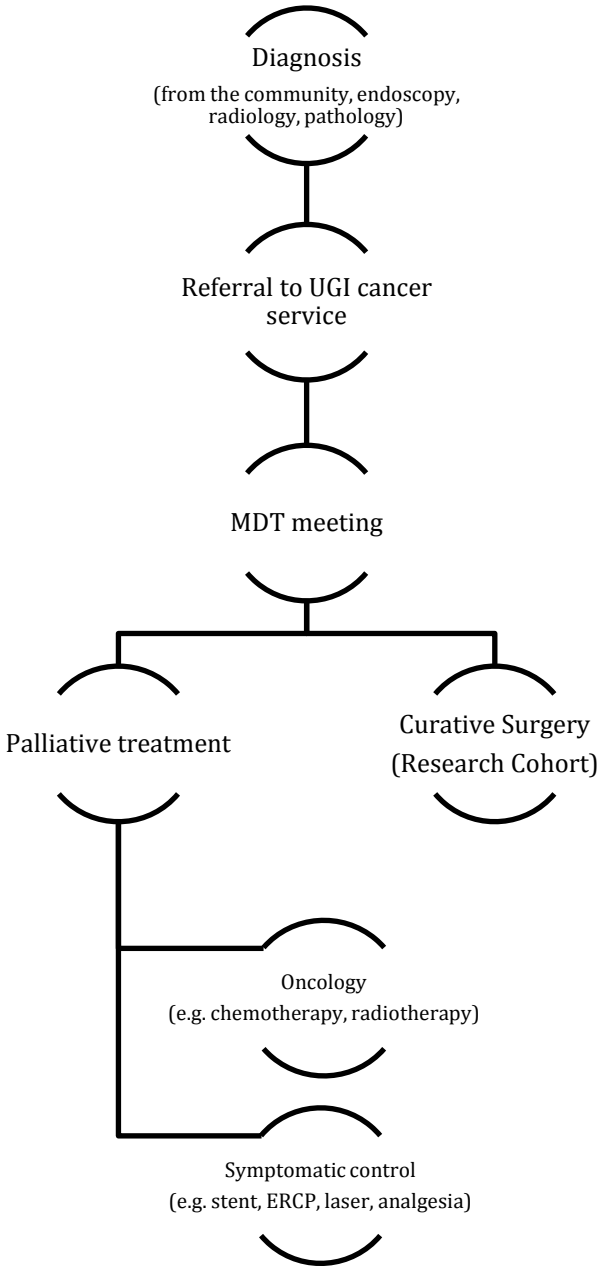
2.1 ***Patient recruitment***

2.1.1 **Patient identification, consent and ethics**

Patients were identified via the UGI cancer multi-disciplinary team (MDT) meetings. These meetings comprised surgeons, physicians, oncologists, nurse specialists, dieticians, pathologists and radiologists and were held weekly to discuss all cancer referrals mainly from the Lothian, Borders and Fife regions along with tertiary referrals from other hospitals in the central belt of Scotland. The meetings were held weekly (one for gastro-oesophageal cancers and another for hepatobiliary cancers) in the Royal Infirmary of Edinburgh. Figure 2.1 illustrates the 'normal' pathway that a patient would progress along once diagnosed with cancer. It is from the surgical cohort that potential patients to be included in the studies outlined in this thesis were identified. Cancer patients discussed at these MDT meetings who were thought to have surgically resectable tumours were subsequently approached at their outpatient clinic appointment. The study was discussed with them and a patient information sheet supplied (see Appendix 1).

Control patients for the muscle biopsy studies (Part I) were recruited from elective theatre lists. Those who were undergoing surgery for benign, non-inflammatory conditions were approached at outpatient clinics or pre-operative assessment clinic. The study was discussed with them and a patient information sheet supplied (see

FIGURE 2.1: THE DIAGNOSIS TO TREATMENT PATHWAY FOR PATIENTS WITH UGI CANCERS



Patients with a suspected or confirmed diagnosis of UGI cancer were referred to the central MDT meeting (comprising surgeons, radiologists, pathologists, nurse specialists, physicians, oncologists, and dieticians). A consensus decision on treatment was then made. Patients included in this thesis were recruited from the Curative Surgery cohort.
Abbreviations: MDT, multi-disciplinary team; UGI, upper gastrointestinal; ERCP, endoscopic retrograde cholangiopancreatography.

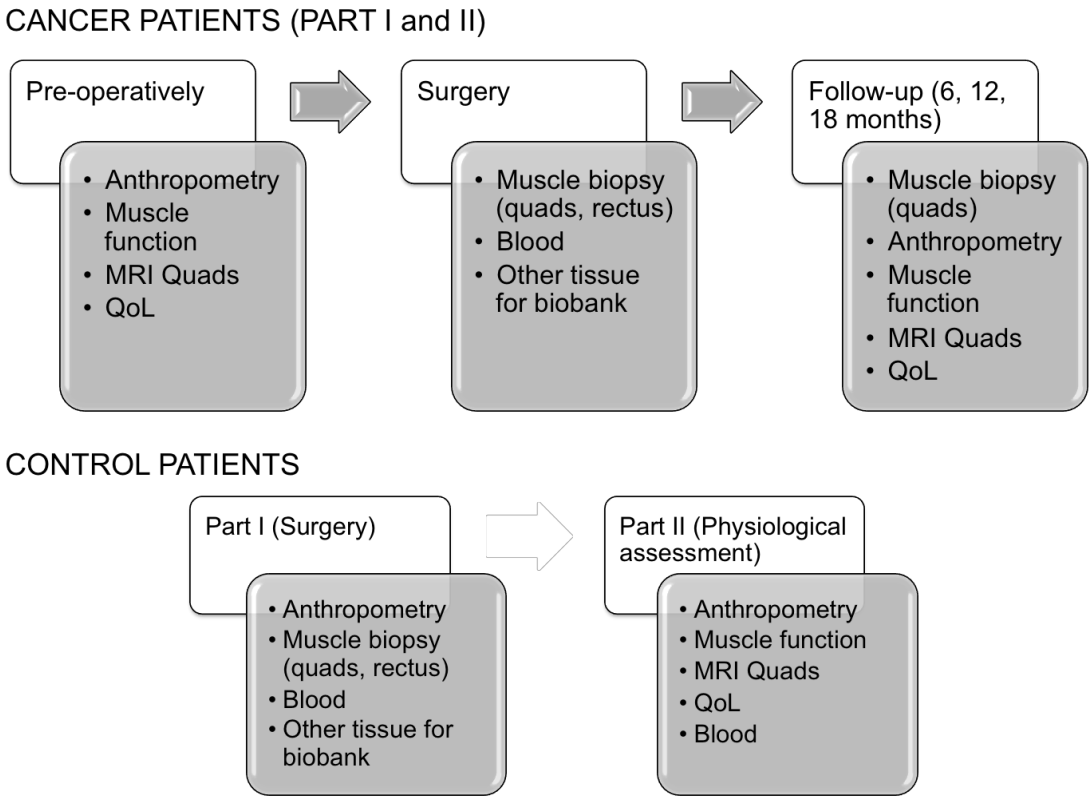
Appendix 1). Control patients for the physiological assessment studies (Part II) were recruited from the community. Adverts were placed in buses and bowling clubs and interested participants were asked a screening questionnaire (see Appendix 1).

All patients were then approached again in person, if they had a further clinic visit, or were phoned to confirm their willingness to participate in the study and to answer any questions. The Clinical Research Facility (housing the muscle function equipment) and an MR scan were then booked to coincide with the patient attending for operation (if arriving the day before surgery) or their pre-assessment visit, which was usually carried out in the week preceding their operation. Patients' were asked about clinical details and these were recorded and confirmed/supplemented with information from the patients hospital records. Written consent was taken prior to entry into the study (see Appendix 1). All procedures were approved by the local research ethics committee (Reference 06/S1103/75). The studies conformed to the standards set by the Declaration of Helsinki.

2.1.2 **Proposed pathway/protocol of investigation**

Following consent, patients underwent a series of anthropometric and muscle function assessments and MR imaging as detailed below. For patients in the muscle biopsy studies, tissue samples were collected at the time of operation. A diagram of the planned patient pathway is shown below (Figure 2.2). Repeat assessments for cancer patients were carried out at around 6, 12 and 18 months following surgery and aimed to tie in with routine post-operative surgical outpatient appointments.

FIGURE 2.2: PLANNED PROTOCOL FOR PATIENT PATHWAY



In cancer patients a repeat quadriceps muscle biopsy was planned at one of the follow up assessments if there was significant progressive weight-loss. Abbreviations: MRI; Magnetic resonance imaging, QoL; Quality of life.

2.2 Anthropometric assessments

2.2.1 Height, weight and weight change

Body weight was measured with participants in light clothing using a beam scale (Seca, UK). Height was measured using a standard wall mounted measure. Weight change was calculated as the percentage difference between stable pre-morbid weight and the weight at assessment. The pre-morbid weight was taken from the clinical notes if available or from patient recalled weight. Whilst this could be subject to recall bias, there is evidence that self-reported weight is reliable (Perry et al., 1995, Stunkard and Albaum, 1981).

2.2.2 Body mass index (BMI)

BMI was calculated according to the formula:

$$\text{BMI (kg/m}^2\text{)} = \text{weight (kg)/ height (m)}^2.$$

2.2.3 Triceps skin fold (TSF) thickness

Callipers (Holtain Ltd Crymych UK) were used to measure the TSF thickness (mm) at the mid-arm point of the non-dominant arm (land-marked according to international standards ((ISAK) guidelines 2001) (Kinanthropometry, 2001).

2.2.4 **Mid-arm muscle circumference (MAMC) and cross-sectional area (CSA)**

Mid-arm circumference (MAC) was measured (at the same site as for TSF above) to calculate MAMC using the formula:

$$\text{MAMC (cm)} = \text{MAC (cm)} - [\pi \times \text{TSF (mm)}]$$

Arm muscle CSA was calculated according to the equation:

$$(\text{MAC} - \pi \times \text{TSF}^2/4\pi) - 10 \text{ (male) or } (\text{MAC} - \pi \times \text{TSF}^2/4\pi) - 6.5 \text{ (female)}$$

(Heymsfield et al., 1982).

2.3 Measures of muscle function

2.3.1 **Hand grip dynamometry**

Patients were seated with the shoulder relaxed, the elbow flexed at 90° and the forearm unsupported. Hand grip strength (kg) was measured using a hydraulic dynamometer (Fabrication enterprises Inc., USA). Three measurements were obtained for each side and the highest value used in further analysis

2.3.2 **Isometric knee extensor strength (IKES)**

Maximum voluntary IKES (Newtons (N)) was measured using an established method (Moulds et al., 1977). The participant was seated in an adjustable straight-backed chair with the pelvis secured and the knee flexed at 90^{o}. A cuff was placed around the ankle and attached via an inextensible chain to a strain gauge and data acquisition system (Powerlab, AD instruments, UK) (Figure 2.3A). Following

instruction, the participant made a maximum voluntary contraction, which was held for 5 seconds. Three separate measurements were obtained for each limb and the highest value used in subsequent analysis. Data were normalised to body weight (N.kg-1).

2.3.3 **Lower limb explosive power (LLEP)**

LLEP (Watts (W)) was measured using the Nottingham Power Rig (Medical Engineering Unit, University of Nottingham, UK) (Bassey and Short, 1990) (Figure 2.3B). The participant was seated on the rig with the seat position adjusted so that in full extension the footplate was fully depressed. The participant pushed as hard and as fast as possible against the footplate to accelerate the flywheel. The final velocity of the flywheel was used to calculate the average power output (W) during a single maximal thrust of the lower limb. The process was repeated five times with each limb and the highest value used in subsequent analysis. Data were normalised to body weight (W/kg).

2.3.4 **Sit-to-stand time (STS)**

As a measure of functional ability, STS was measured. Patients were seated and the time taken to rise from a seated to standing position without the aid of their arms was recorded. The best (i.e. quickest) of three measurements was used for analysis.

FIGURE 2.3: EQUIPMENT USED FOR ASSESSMENT OF LOWER LIMB MUSCLE FUNCTION

A.

Ankle bracelet attached to strain gauge at back of frame.

B.

*(**A.**) Chair, frame and strain gauge used to measure isometric knee extensor strength. (**B.**) Nottingham Power Rig used to measure lower limb explosive power.*

2.3.5 **Three metre timed up-and-go (TUG)**

As an additional measure of functional ability, TUG was measured. Two points were marked on the floor 3m apart. Chairs were placed on each of these marks. Patients were seated on one of the chairs and asked to walk around the other chair and back to the original seat; a 6m journey in total. Patients were observed for the entire journey to ensure they walked and did not run (if this did occur, the measure was cancelled and a further measurement taken). The total time was recorded and the best of three measurements was used for analysis.

2.4 Quality of life assessment (QoL)

2.4.1 **Karnofsky Performance Score (KPS)**

KPS was assessed in each patient by one observer. Appendix 1 outlines the scoring system (Karnofsky DA, 1949).

2.4.2 **European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire Core 30 (EORTC QLQ-C30)**

EORTC QLQ C-30 (Aaronson et al., 1993) was assessed in cancer patients and results analysed using linearised scores (scoring manual, version 3). The questionnaire is outlined in Appendix 1.

2.5 Direct measurement of physical activity

2.5.1 Use of the ActivPAL™ monitor

Physical activity and sedentary behaviour were recorded continuously over a three-seven day period using an ActivPAL™ activity monitor (Figure 2.4) (PAL technologies Ltd., Glasgow, UK). ActivPAL™ is about half a credit card size accelerometer-based activity monitor worn on the anterior aspect of the thigh. This instrument gives time stamped information every 0.1s on posture (sitting/lying, standing or walking/running) and walking cadence. It has been shown to be valid, accurate and sensitive in older adults (Grant et al., 2008) and in cancer patients (Dahele et al., 2007, Maddocks et al., 2010). Patients were instructed regarding use of the monitor and the protocol for bathing/showering.

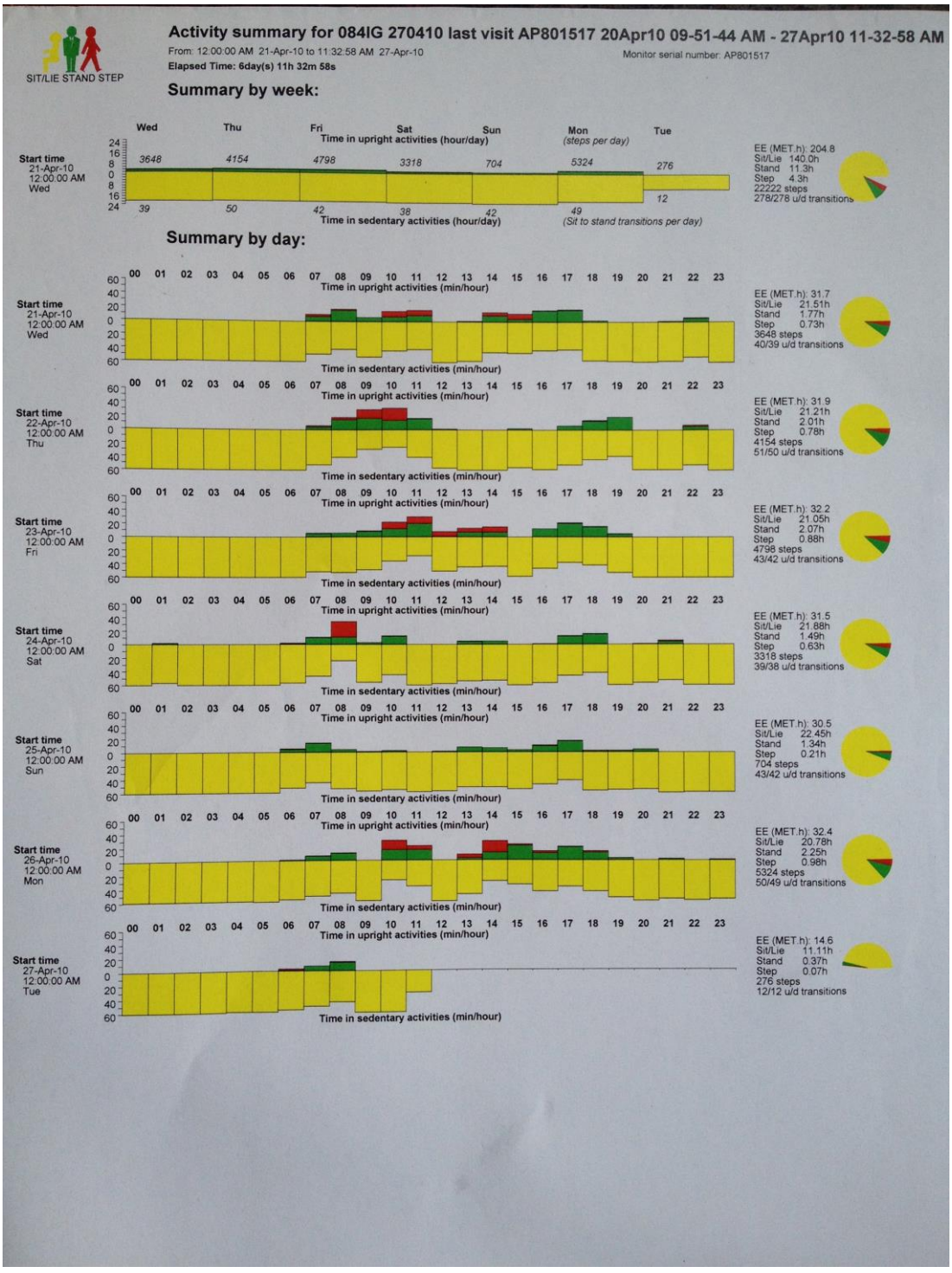
2.5.2 Analysis of ActivPAL™ results

As soon as the study time period was over, the monitor was removed from the patient and data downloaded through the Universal Serial Bus (USB) portal using the ActivPAL^{TM} software provided. The results were entered into an Excel® (Microsoft Corporation) spreadsheet for further analysis. The range was a minimum of 3 and up to 7 days of data. The output gives details of number of steps, up/down transitions, time spent sitting/lying, time spent standing, time spent stepping along with an estimate of energy expenditure (Metabolic Equivalent of Task (MET)) for each 24 hour period. An example of this output summary is shown in Figure 2.5.

FIGURE 2.4: THE ACTIVPAL™ MONITOR

The ActivPAL™ monitor was attached to a patient's anterior thigh using adhesive dressings.

FIGURE 2.5: THE ACTIVPAL™ DATA OUTPUT



ActivPAL™ data output summary sheet. Colour key is in the top left corner. Abbreviations: EE, energy expenditure; MET, metabolic expenditure of task; u/d, up/down.

2.6 Radiological imaging

2.6.1 MR imaging

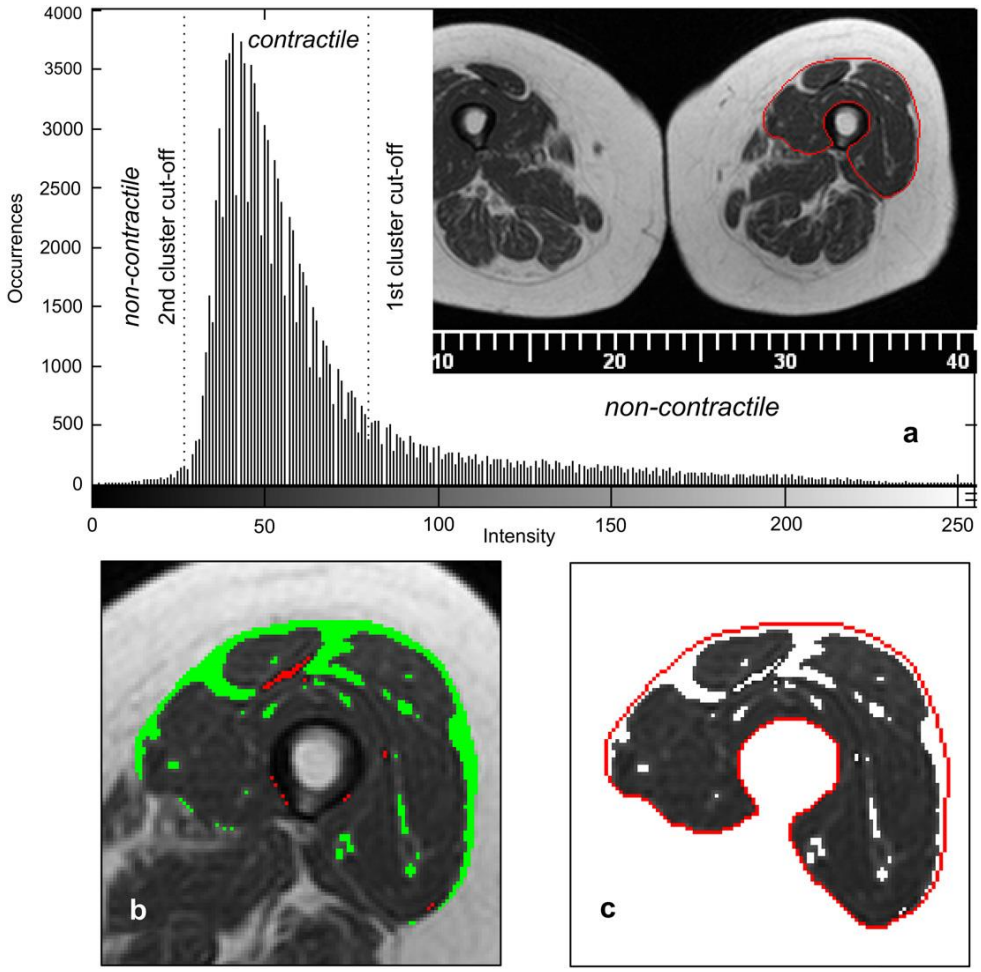
2.6.1.1 MR scan protocol

Participants lay supine within the 1.5 Tesla MR Imaging system (Phillips Gyroscan Intera, The Netherlands) using a Q-body receiving coil with the system isocentre located at the mid-femur point where an oil-capsule was placed on the skin for reference (land-marked prior to the scan according to international standards ((ISAK) guidelines 2001) (Kinanthropometry, 2001). Longitudinal relaxation time (T1)-weighted spin echo axial images were prescribed from the proximal border of the patella to the superior anterior iliac spine. Imaging parameters were: slice thickness 10 mm (no gaps); acquisition matrix 512 × 512; field of view 500 mm; echo time 15 ms; repetition time 425 ms; and flip angle 90°. Three acquisitions were obtained to improve the signal to noise ratio. The number of slices acquired ranged from 38 to 45 depending on thigh length. The legs were immobilised and supported using strapping during data acquisition to minimize motion artefact. Based on visual inspection of the images, no motion artefact was apparent. The total scan time was 6 minutes.

2.6.1.2 Analysis of MR images

The CSA of the thigh muscle was quantified on each image by drawing a region of interest (ROI) around the quadriceps muscle using the biomedical imaging software package ANALYZE 8.0 (Mayo Clinic, Rochester, USA) (Figure 2.6). This

FIGURE 2.6: THE APPLICATION OF K-MEANS STATISTICAL TECHNIQUE TO MR SCANS



(a, inset) *Single MR slice (cropped display) taken at mid-thigh level. The manually drawn region of interest (ROI) comprising the quadriceps muscle group (left leg) is outlined in red. The posterior boundary of the ROI excluded closely located muscles (i.e., Sartorius, Adductor longus and the short head of Biceps femoris).* ***(a)*** *Histogram representing the intensity distribution for pixels lying within the ROI for the whole volume. Resulting cut-off values between contractile and non-contractile values are also shown for each application of the k-means clustering algorithm.* ***(b)*** *K-means processed image for the ROI shown in* ***(a)****. Voxels tagged as the high intensity non-contractile component after the 1st clustering application are highlighted in green, those tagged as the low intensity non-contractile component found after the 2nd clustering application are highlighted in red. The ROI boundary shown in red in* ***(a)*** *has been removed for clarity.* ***(c)*** *Resulting optimized contractile tissue component segmented from ROI. The ROI's shown in* ***(b)*** *and* ***(c)*** *have been cropped and magnified for illustration purposes.*

initial quantification of the muscle cross-sectional area is dependent upon the anatomical knowledge and manual segmentation skills of the operator. Intra- and inter-investigator reliability (using 6 whole leg scans i.e. ~270 images) for segmentation was measured using Bland Altman analysis with plots of individual slice volumes demonstrating maximum differences within and between (n = 2) raters of no greater than 0.4% (3.8 cm^3) when expressed as a percentage of a total gross muscle volume of 1 litre. For studies utilising an individual cross-sectional slice, the mid-femur point was chosen (identified on scan with the oil-capsule). Volume measurements were obtained by multiplying area measurements by slice thickness for all cross-sectional slices comprising quadriceps muscle. Values were adjusted for height (CSA divided by height squared) (VanItallie et al., 1990).

2.6.1.3 **MR image analysis and muscle optimization using k-means statistical clustering**

The manually outlined ROI for each slice of the quadriceps group were converted to binary mask images. Software developed in matrix laboratory (MATLAB) version 7.6 (R2008a, The Mathworks Inc., USA) combined all the MR images into a single array and similarly the ROI binary mask images were also combined into a single image array. The two arrays were then multiplied so that all areas outwith the quadriceps muscle region were masked during cluster analysis. This single masked MR image array was created for processing with the k-means algorithm so that a global range for voxel intensity values could be determined (i.e., intensity-based clustering was performed across the whole MR scan rather than on a slice-by-slice

basis to reduce the impact of localised image heterogeneity).

Cluster analysis using the k-means algorithm was performed on the masked MR image array in a two step procedure. The number of clusters is decided by the operator, subsequent steps are operator independent therefore reducing bias. The first application of the k-means algorithm separates the entire quadriceps muscle region into 2 clusters by minimizing the cluster variance for the high and low signal intensity peaks: i.e. partitioning high signal component (light pixels representing fat) and low signal (dark pixels representing muscle, bone, tendon). The grouping is done by minimizing the intra-cluster variance (sum of squared distances) between data points and the corresponding cluster centroid according to the following equation:

$$V = \sum_{i=1}^{k} \sum_{x_j \in S_i} (x_j - \mu_i)^2$$

where there are k clusters S_i (i = 1, 2,…, k), (k = 2 in the present implementation), x_j is the intensity value of an incoming j^{th} pixel, and μ_i is the centroid (mean intensity value) of the i^{th} cluster. The second application of the k-means algorithm is applied to the low signal cluster containing the optimized muscle. This is a refinement operation that separates the dark pixels (muscle tissue) from the very dark pixels (bone, tendon). During the manual outlining of the quadriceps muscle region, the operator may include inadvertently pixels representing the femur bone due to the discrete nature of the image, therefore a second application of the k-means algorithm further aids optimization of the muscle region by excluding obvious non-muscle components. The output cluster map was used to reconstruct a k-means optimized

muscle MR image array, which was segmented using a 512 × 512 image matrix to reform individual images (Figure 2.6). The pixels representing optimized muscle within each MR slice were summed to provide a measurement of CSA and total quadriceps volume. This process was repeated for each of the remaining cluster maps created during the two applications of the k-means algorithm, giving a measurement of high and low signal non-muscle components. The gross quadriceps volume was calculated from the sum total of the cluster volumes.

2.6.2 **Computed tomography (CT) scans**

2.6.2.1 **Obtaining CT scans**

CT scans were performed as part of the cancer patients' routine management but were not available for the benign controls. Initially the CTs were manually downloaded onto compact discs for subsequent analysis. During the study period, the hospital introduced an online radiology system, which allowed direct access to the scans online and the ability to save the image directly.

2.6.2.2 **Analysis of CT scans**

The CSA of skeletal muscle and adipose tissue (comprising subcutaneous, visceral and intermuscular) was measured at the level of the 3rd lumbar vertebrae (L3). Adipose and muscle CSA measured at L3 are linearly related to whole body values (Mourtzakis et al., 2008). All images were analysed by a single trained observer with SliceOmatic V4.3 software (Tomovision, Montreal, Canada), which enables

specific tissue demarcation using Hounsfield unit (HU) thresholds. The HU ranges used were -29 to +150 for skeletal muscle (Mitsiopoulos et al., 1998), -190 to -30 for subcutaneous and intramuscular adipose tissue (Kvist et al., 1986) and -150 to -50 for visceral adipose tissue (Vehmas et al., 1996). Tissue boundaries were corrected manually if required. CSAs (cm^2) were computed automatically by summing tissue pixels, multiplying by pixel surface area and subsequently normalised for stature (cm^2/m^2).

Routine diagnostic CT scans usually only evaluate the chest, abdomen and pelvis and therefore only partial images are available to determine skeletal muscle mass. Estimates of whole body stores were generated from the raw data (cm^2) using the following regression equations (Mourtzakis et al., 2008) which show a close correlation between muscle and fat areas in CT images at L3 and whole body compartments of FFM and fat mass (FM) respectively.

- Total body FFM (kg) = 0.3 x [skeletal muscle at L3 (cm^2)] + 6.06, (r = 0.94)
- Total body FM (kg) = 0.042 x [total adipose tissue at L3 (cm^2)] + 11.2, (r = 0.88)

2.7 ***Mechanical muscle quality***

MR imaging derived mid-femur muscle CSA was combined with the functional measurements to give a measure of muscle mechanical quality according to the formula: Muscle Quality (N/mm^2) = Strength (IKES)/ CSA.

2.8 Tissue sampling and storage (establishing the biobank)

2.8.1 Blood

2.8.1.1 Collection of blood sample

A fasting blood sample was taken on the morning of surgery. This was either venous (peripherally or through a central line if present), or arterial (through arterial blood pressure monitoring line) depending on availability according to the magnitude of operation that the patient was going to have.

At follow-up appointments, patients were asked to fast overnight prior to their assessment. A venous blood sample (~12ml divided into 3 tubes) was then taken from the anticubital fossa. Pressure was applied till haemostasis was achieved and then a plaster was put over the wound.

2.8.1.2 Blood sample preparation and storage

Samples were collected in lithium heparin tubes. One tube was delivered to the hospital (RIE) biochemistry lab for further analysis. Normal reference ranges are outlined in Table A10, Appendix 1. The other two each had 1.5ml whole blood withdrawn and stored in tubes and then put in a centrifuge cooled to 4°C and spun at 15000 revolutions per minute (rpm) for 15 minutes. The supernatant (plasma) was pipetted into tubes and stored at -80°C. The fluid beneath the 'bead line' were red cells and these were also pipetted into tubes and stored at -80°C.

2.8.2 *Rectus abdominis* muscle

2.8.2.1 Collection of *Rectus abdominis* muscle biopsy

All biopsies were taken at the start of open abdominal surgery. The edge of the *Rectus abdominis* was exposed and a ~1cm^3 specimen removed using sharp dissection. The specimen was cleaned of any gross blood contamination and any visible fat/fibrous tissue removed. The specimen was divided for four purposes:

- *Raw muscle*: A section of muscle was placed in a tube (NUNC® Cryotubes®, Sigma-Aldrich Co. LLC) and snap frozen in liquid nitrogen before storing at -80°C.
- *Cryo-section*: 5ml of isopentane (Sigma-Aldrich Co. LLC) was cooled in a tube bathed in liquid nitrogen. Once the solvent became viscous and the edges started to solidify white (temperature approaching -190°C), the muscle was introduced to allow freezing. This was achieved by stitching a section of muscle to a cork disc using Vicryl™ (Ethicon Endo-Surgery Inc.) and Optical Cutting Temperature (OCT™) medium (Tissue-Tek®, Sakura Finetek, Europe) was placed at the base of the muscle. The specimen was lowered with the cork uppermost (i.e. muscle first) into cooled solvent and held for approximately 5 minutes (until frozen). This was then stored in a separate tube at -80°C.
- *Paraffin block*: A section of muscle was cut and placed in formaldehyde (10% formalin solution). This was left for 24 hours before removal and placing in a series of ethanol dilutions and delivering to the University of Edinburgh pathology lab for mounting into paraffin blocks.

- *Electron microscopy*: A thin segment of muscle was dissected/teased out. This was mounted on a wax strip and pinned with a sterile needle. This was then placed in fixative (3% glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH 7.3) for 2 hours before further processing (Section 2.11).

2.8.3 **Quadriceps muscle**

2.8.3.1 **Collection of quadriceps muscle biopsy**

Baseline

At the time of resectional surgery, after induction of anaesthesia, iodine solution was used to clean the skin. A 0.5cm skin incision was made with a number 15 scalpel blade and deepened to the muscle fascia over *Vastus lateralis* muscle bulk at the level of the mid-femur point. A Conchotome forceps (Figure 2.7A) was used to obtain a muscle biopsy from this site. Three bites were taken and 5 minutes of direct pressure was subsequently applied to achieve haemostasis. The skin wound was closed with Steri-Strips™ and Tegaderm™ dressing (both 3M Nexcare™).

Follow-up

Patients were fasted overnight prior to the follow-up biopsy, which was carried out in the morning. The procedure was as for the baseline biopsy except for the following points: 5ml 1% lignocaine local anaesthesia was infiltrated at the site of biopsy prior to skin incision and a Bergstrom needle (Figure 2.7B) was used for the biopsy which was taken under suction to improve tissue yield. The Bergstrom was used because on initial attempts at biopsy with the Conchotome under local anaesthesia, patients

reported increased pain and bruising after the biopsy. This was not an issue with use of the Conchotome whilst under general anaesthesia.

2.8.3.2 **Quadriceps muscle biopsy preparation and storage**

Gross blood and any fat/fibrous tissue were removed. The biopsy was divided into two pieces (one for protein, one for RNA extraction) and placed in a tube (NUNC® Cryotubes®, Sigma-Aldrich Co. LLC), snap frozen in liquid nitrogen and then stored at -80°C.

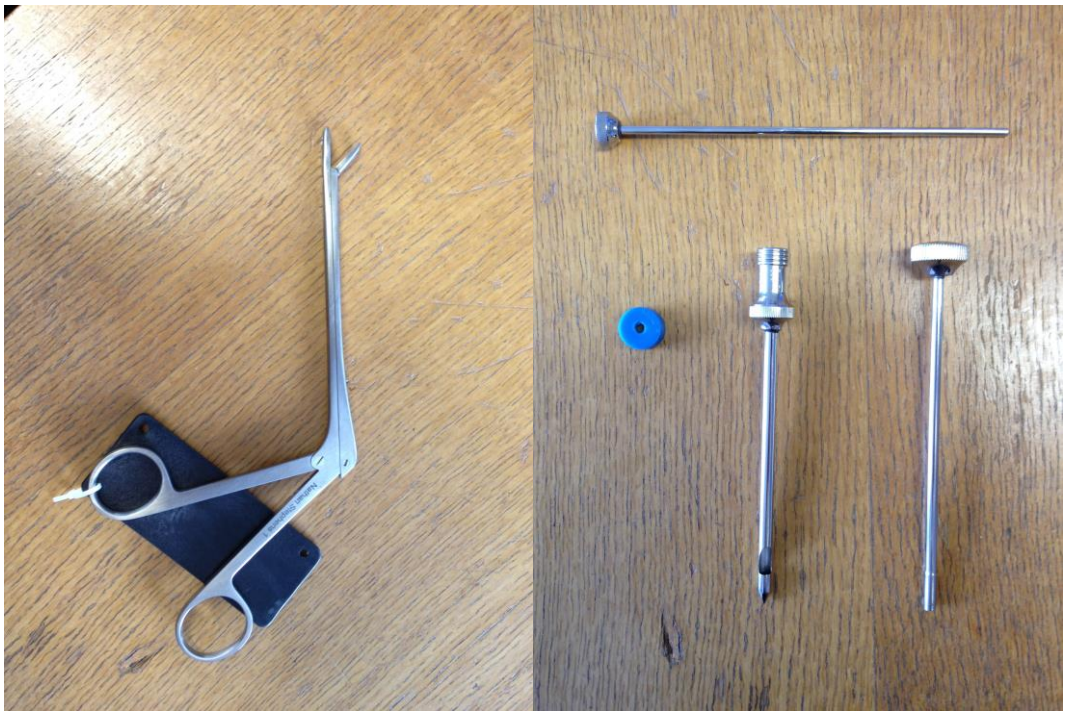
2.9 Blood measures

2.9.1 **Clinical Chemistry Laboratory, RIE**

All samples were processed in the Department of Clinical Chemistry, RIE (fully accredited by Clinical Pathology Accreditation Ltd, UK): Haemoglobin was measured using an automated analyser (Sysmex XE-2100), CRP by ELISA (Ely, UK), sex-hormone binding globulin (SHBG) (DPC Immulite) and albumin (Olympus 640). Total testosterone (TT), oestradiol, luteinising hormone (LH), follicle stimulating hormone (FSH), insulin and cortisol were measured using standard automated methods (Bayer Immuno-I) following the manufacturer's instructions. Calculated free testosterone (cFT) was calculated according to the Vermeulen formula (Ho et al., 2006).

FIGURE 2.7: THE CONCHOTOME AND BERGSTROM BIOPSY NEEDLES

A. B.



1 ***2*** ***3***

*Percutaneous Vastus lateralis muscle biopsies were taken using either the Conchotome forceps (**A.**) or the Bergstrom needle (**B.**). The Conchotome biopsy was performed by opening and closing the forceps handles. For the Bergstrom needle, part 2 was inserted through the rubber bung (1) and into part 3. Suction was applied using a syringe and plastic tubing inserted into the top of the needle. The component at the top of the picture was used after the biopsy to retrieve the muscle tissue from within the needle.*

2.10 ***Skeletal muscle measures***

2.10.1 **Cutting of raw muscle sample**

Raw muscle was placed on a piece of aluminium foil previously cooled over dry ice. This was placed on a slate covered in foil and resting in a container of dry ice. A razor blade and forceps were cooled on dry ice. The forceps and razor blades were cleansed in pure ethanol and wiped clean until no macroscopically visible tissue was evident. Instruments were discarded after three uses and a new sterile autoclaved forceps and a new razor blade used. Samples that had been cut were placed back in their storage tube and into the -80°C freezer as soon as was feasible thereafter.

2.10.2 **Whole protein isolation**

Approximately 20mg of frozen tissue was homogenised in 0.5ml of lysis buffer (Triton X-100 (1%), NaCl (150mM), Tris-HCl (50mM), EDTA (1mM), PMSF (1mM), protease inhibitors (Roche Diagnostics Ltd.) (1 tablet per 10ml), water to 10ml) using a Powergen 125 (Thermo Fisher Scientific Inc.) electric homogeniser. Samples were left on ice for 15 minutes prior to centrifuging at 13000 rpm for 15 minutes. The supernatant was removed, and samples were then stored at -80°C.

2.10.3 **Nuclear/cytoplasmic protein extraction**

Approximately 20 mg of muscle was re-suspended in 180 µl of low salt lysis buffer (10mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, protease inhibitors (Roche Diagnostics Ltd.) (1 tablet per 10

ml)) and ground using a handheld glass homogeniser. Samples were incubated on ice for 5 minutes before two cycles of freeze-thaw lysis. After a brief vortex, samples were centrifuged at 4000 rpm for 3 minutes. The supernatant was removed (representing the cytoplasmic portion which was divided into aliquots and stored at -80) and the pellet (containing the nuclei) re-suspended in 40 µl high salt extraction buffer (20mM HEPES, 420mM NaCl, 1mM EDTA, 1mM EGTA, 25% Glycerol, 1mM DTT, protease inhibitors (Roche Diagnostics Ltd.) (1 tablet per 10ml). Samples were incubated on ice for 30 minutes with gentle mixing of the tubes every 5-10 minutes. Samples were centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant (containing the nuclear proteins) was divided into aliquots and stored at -80°C.

2.10.4 **Determination of protein concentration**

The tissue homogenisation buffer used for extraction of protein from muscle was used to prepare a set of dilutions from a control protein (bovine) as follows:

A – neat standard
B – 1 in 2
C – 1 in 4
D – 1 in 8
E – 1 in 16
F – blank

Each set of standards and samples was done in triplicate using an immunoassay plate. Protein solutions from the patients were diluted to 1 in 5, 1 in 10 and 1 in 20 using tissue homogenisation buffer. Reagent was added to each well and the plate shaken for at least 15 minutes to allow colour to develop. The plate was placed in a plate-

reader to determine protein concentration. Results from the control protein were inspected to ensure a good correlation value and then the volume for the given amount of protein calculated for each of the patient samples. If the calculations for the final concentration for each sample dilution (i.e. 1 in 5, 1 in 10, 1 in 20) was not similar the plate was discarded and repeated.

2.10.4 **RNA isolation**

Total RNA was extracted from approximately 20mg of muscle using TRIzol (Invitrogen, UK) reagent according to the manufacturer's directions. The RNA pellet was re-suspended in DEPC treated water and RNA concentration was determined using a Nanodrop spectrophotometer (LabTech International, UK). RNA quality was assessed using 260/280, 230/260 ratios and the RIN score from the BioAnalyzer 2100 instrument (Agilent Technologies).

2.10.5 **Western blotting (immunoblotting)**

2.10.5.1 **Gel preparation**

Gels were hand poured to the required percentage: separating gel (0.375M Tris, ph 8.8) and stacking gel (0.125M Tris, pH 6.8). Alternatively, pre-poured fixed gradient percentage gels (4-12%) were used (NuPAGE Novex 4-12% Bis-Tris gradient gels, Invitrogen, Paisley, UK).

2.10.5.2 **Western blotting protocol**

20µg of protein from each sample was added to 3µl of 4 x LSB (0.5M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.05% β-mercaptoethanol, 0.004% bromophenol blue) and boiled for 3 minutes. Proteins were resolved using SDS-polyacrylamide gel electrophoresis at 160V for 45 minutes. Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Ltd, Bucks, UK) using semi-dry transfer (80mA for 1 hour). Membranes were blocked with either 3% BSA/TBST (TBS, 0.05% Tween) overnight at 4°C or with 5% milk/TBST for 1hr at room temp. Incubation with primary antibody (1:1000) was carried out in either 3% BSA/TBST or 0.5% milk/TBST solution at room temperature for 2 hours or overnight at 4°C. Membranes were washed with TBST and primary antibody binding detected using horseradish-peroxidase conjugated secondary antibodies (1:2000 to 1:5000). Specific signal was detected using Amersham ECL reagent (GE Healthcare Life Sciences) and exposure on photographic film (Kodak Ltd., UK).

2.10.5.3 **Antibodies used**

The primary antibodies used in the study were Akt, pAkt (Ser473), FOXO1, FOXO3a, phos-CAMk2(Thr286) (Cell Signaling), Lamin A/C (Santa Cruz Biotechnology Inc), alpha-skeletal actin (Novocaestra), CAMk2 (BD Biosciences); dystrophin (MANDYS102 (7D2)), β-dystroglycan (MANDAG2 (7D11)), (Developmental Studies Hybridoma Bank, University of Iowa); β-sarcoglycan (Abcam); myosin heavy chain (fast) (Sigma-Aldrich Co. LLC).

Secondary antibodies were horseradish-peroxidase conjugated secondary antibodies (1:2000 to 1:5000) (anti-mouse, anti-rabbit: Upstate).

2.10.5.3 **Western blot image analysis/densitometry**

Photographic films (Kodak Ltd) were scanned onto a computer. Densitometry values were estimated using ImageJ (NIH) software.

2.10.6 **Quantitative real time polymerase chain reaction (qRT-PCR)**

2.10.6.1 **Primer design and sequences used**

Primers were designed to span introns using Primer Express 3.0 software (Applied Biosystems) and constructed by Invitrogen (Paisley, UK). Primers are shown in Table 2.1.

Table 2.1: Primer sequences

A.

Gene	Forward Primer	Reverse Primer
APCDD1	*GCA CCG AGT TCG TGT TCA A*	*TCC CGT TGA AGA CGT TGA G*
BNIP3	*GTC AAG TCG GCC GGA AAA TA*	*GCG CTT CGG GTG TTT AAA GA*
CAMK2β	*GAC GGC AAG TGG CAG AAC*	*CAA CTC TGT CCG GCG AAA*
EIF3I	*GAA GCC ATG GAT GTA ACC ACA*	*AGG TCC AAA GTG ACC CTT GA*
GABARAPL1	*CCA CCG CAA GGA GAC AGA AG*	*GAA AAT GTG ATG ACG GTG TGT GT*
HGS	*ACA GAC TCT CAG CCC ATT CC*	*AGA CTC GCC ATT GTG GAA CT*
MAFBX	*CCG GCT GTT GGA GCT GAT A*	*TTG GGC GAT GCC ACT CA*
MURF1	*GCT AGG CGT GGC TCT CAT TC*	*TCC TGG ATC AGG CTC GAC TT*
NUDC	*AAG AAC GGC AGC CTT GAC T*	*GTC CTT CTC ATC TTC CTC CTC A*
POLRMT	*AGA CCA AGA CCG CAG GAA G*	*CTC CGA CAC GCT CTC AGC*
SGK1	*TTT CCA AAG AGG GGT TCT CC*	*TGG CAT GAT TAC ATG GCT CT*
TIE1	*GCC CAG ATT GCG CTA CAG*	*ATC AAT GCC CGC GTA AGT*
TSC2	*GCA GCA TCA GTG TGT CTG AAC*	*AAG CTG GCA CTG GTG AGG*

B.

Gene	Forward Primer	Reverse Primer
COMP	*GAT CAC GTT CCT GAA AAA CAC G*	*GCT CTC CGT CTG GAT GCA G*
ADIPOQ	*TAT CCC CAA CAT GCC CAT TCG*	*TAG GCA AAG TAG TAC AGC CCA*
MMP3	*ATG GAC AAA GGA TAC AAC AGG GA*	*TGT GAG TGA GTG ATA GAG TGG G*
PCK1	*CAA GAC GGT TAT CGT CAC CCA*	*GAA CCT GGC ATT GAA CGC TT*
ANGPTL7	*CCA GAG ACG AAA AAG TGG CCT*	*GAG AGC CGG TGG ATG TGT T*
HSP90AB1	*GAC TTG TGT CTT CAC CTT GCT*	*GGT GGA GTT GTC CCG AAG TG*
SLC25A37	*AAG ACC CTT CTG AAC ACT CAG G*	*GTT GAG CTG GTA CAC CGT CC*
PROX1	*TTG ACA TTG GAG TGA AAA GGA CG*	*TGC TCA GAA CCT TGG GGA TTC*
RCAN1	*GGC GAC TGG AGC TTC ATT GA*	*CAT ACG TCC TAA AGA GGG ACT CA*
HINT3	*AGA GCC CAA GGA CTA CGA CAG*	*GCT TCT TTG GCA CCA CAA GAT A*
BNIP3	*AAC ACG AGC GTC ATG AAG AAA GGG*	*ATC CGA TGG CCA GCA AAT GAG AGA*
GABARAPL1	*CCA CCG CAA GGA GAC AGA AG*	*GAA AAT GTG ATG ACG GTG TGT*
MuRF1	*CTT CCA GGC TGC AAA TCC CTA*	*ACA CTC CGT GAC GAT CCA TGA*
MAFBx	*TCC AGA CCC TCT ACA CAT CCT*	*AGA ATC GTC TCC ATC CGA TAC A*

*Primers were designed using Primer Express 3.0 software (Applied Biosystems). (**A.**) Primers used in the cross-sectional Rectus abdominis muscle transcriptomic study (Chapter 4). (**B.**) Primers used in the longitudinal Vastus lateralis muscle transcriptomic study (Chapter 5).*

2.10.6.2 **qRT-PCR protocol**

Total RNA was extracted as described in Section 2.10.4. cDNA was prepared using 1µg RNA, TaqMan reverse transcription reagents (Applied Biosystems) and random hexamer primers (Applied Biosystems). Primers were designed and used as above. Samples were run on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems) in triplicates of 20 µl per well using SYBR Green PCR Master Mix (Applied Biosystems) as per manufacturer's instructions. Expression levels were normalised to ribosomal 18S RNA. Results were analysed using the ΔCt method (Livak and Schmittgen, 2001).

2.10.7 **Microarray target preparation and hybridisation**

3.5µg of total RNA was reverse transcribed and processed according to the protocol provided by Affymetrix for the GeneChip Expression 3' Amplification One-Cycle Target Labelling and Control Reagents kit (Affymetrix Inc.). Reverse transcription and second strand cDNA synthesis were followed by in vitro transcription and biotinylation. Biotinylated cRNA products were cleaned up using columns (Affymetrix). The quality of the biotinylated cRNA was assessed by Nanodrop (LabTech International, UK) and BioAnalyzer (Agilent Technologies) instruments and the cRNA was then fragmented according to Affymetrix protocols. Samples were hybridised to the HGU-133plus2 GeneChip array (covering ~54,000 sequences).

2.11 Transmission electron microscopy (TEM)

2.11.1 **Sample preparation**

After fixation for 2 hours (3% glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH 7.3), specimens were washed in three 10-minute changes of 0.1M Sodium Cacodylate. Specimens were then post-fixed in 1% Osmium Tetroxide in 0.1M Sodium Cacodylate for 45 minutes and washed in three 10-minute changes of 0.1M Sodium Cacodylate buffer. These sections were then dehydrated in 50%, 70%, 90% and 100% normal grade acetones for 10 minutes each, then for a further two 10-minute changes in analar acetone. Samples were then embedded in Araldite resin. Sections, 1μm thick were cut on a Reichert OMU4 ultramicrotome (Leica Microsystems (UK) Ltd, Milton Keynes), stained with Toluidine Blue and viewed in a light microscope to select suitable areas for investigation. Ultrathin sections, 60nm thick were cut from selected areas, stained in Uranyl Acetate and Lead Citrate.

2.11.2 **TEM image capture and analysis**

Sections were viewed in a Phillips CM120 Transmission electron microscope (FEI UK Ltd, Cambridge, England). A Gatan Orius CCD camera (Gatan UK, Oxon, England) set at 2650x magnification was used to capture images. Photographs were taken at random and lipid droplets were counted manually (by one observer blinded to the patient diagnosis) in at least two fields per patient and the average count recorded. The diameter of each visible lipid droplet was measured in μm using ImageJ (NIH) and the mean lipid droplet diameter calculated for each patient. Identified lipid droplets were examined at high magnification (11000x) to ensure the

absence of a double membrane, thus distinguishing them from vacuolated giant mitochondria (Gdynia et al., 2010).

2.12 ***Statistical analysis***

2.12.1 **General analysis**

Statistical Programme for Social Sciences (SPSS) v15.0 (IBM) or GraphPad InStat (Graphpad Software Inc.) was used for data analysis. Student's two-tailed t test or one-way ANOVA were used to compare means between groups. Tukey's honest significance test was used for post-hoc analysis. The Mann-Whitney test was used for non-parametric analysis. Data were log transformed when appropriate. Associations between variables were evaluated using Pearson's correlation (bivariate) analysis. Contingency tables were constructed where relevant and analysed by Fishers exact test or Chi-squared test. Univariate ANOVA was used to investigate the effects of covariates on dependent variables and to establish effect size using partial eta-squared. Statistical significance was set at a two-tailed p value of ≤ 0.05.

2.12.2 **Microarray statistical analysis**

Microarray data was analysed using the Microarray Suite software (MAS) 5.0 (Affymetrix). To improve the accuracy of the gene to probe relationship, a custom chip definition file (Sandberg and Larsson, 2007) was used defining the Affymetrix probes by Ensembl transcript ID. Data were normalised using MAS and robust multi-array average (Irizarry et al., 2003). Genes called absent on every array by the

MAS5 software were filtered from the data and remaining genes analysed using quantitative function in significance analysis of microarrays (SAM) (Tusher et al., 2001) implemented in the bioconductor suite (Gentleman et al., 2004). Percentage weight-loss or systemic inflammation (CRP level) were the quantitative variables. To test the robustness of the approach, the limma package (GK, 2005) in the Bioconductor suite was used to identify genes co-varying with weight-loss or systemic inflammation. Both SAM and limma generate a false discovery rate (FDR) (Benjamini Y, 1995). All genes identified by both procedures with a FDR<10% which covaried with weight-loss were further examined. Functional annotation of these genes was carried out using Gene Ontology (GO) (Ashburner et al., 2000) utilising the topGO tool (Alexa et al., 2006) in the bioconductor suite along with web based Ingenuity Pathway Analysis (Anaylsis). For analysis of microarray data the bioconductor suite (Gentleman et al., 2004) and the R language for statistics (R Development Core Team) (version 2.7.1) were used.

Part I: **Cancer cachexia classification: molecular biomarker discovery**

"…in the beginning of the malady it is easy to cure but difficult to detect, but in the course of time, not having been either detected or treated in the beginning, it becomes easy to detect but difficult to cure."

Niccolo Machiavelli *'The Prince'*

"The journey to discover useful biomarkers will require imaginative exploration, fastidious validation and some good luck"

Ransohoff, Science 2003

Chapter 3: **Evaluation of potential biomarkers in human skeletal muscle cancer cachexia; a comparison with pre-clinical models**

3.1 ***Abstract***

Several potential candidate biomarkers were evaluated in skeletal muscle biopsies from a cohort of UGI cancer patients. A total of 107 patients (15 weight-stable healthy controls (HC), 92 UGI cancer patients) were recruited. Cachexia was defined as weight-loss ≥5%. Rectus abdominis muscle was obtained at surgery and analysed by Western blotting or qRT-PCR for Akt/phosphorylated-Akt, FOXO1/3a, MAFbx, MuRF1, BNIP3, GABARAPL1, myosin heavy-chain (MyHC), dystrophin, β-dystroglycan and β-sarcoglycan. Patients were followed up for an average of 3 years or until death. β-dystroglycan levels were higher in skeletal muscle from cachectic compared with non-cachectic cancer patients (p=0.007). The skeletal muscle from cancer patients compared with HC had reduced total Akt protein (p=0.001), increased ratio of phosphorylated to total Akt (p=0.002) and increased expression of GABARAPL1 (p=0.024). Survival was shortened in patients with low MyHC levels or low dystrophin levels (p=0.023 and p=0.008 respectively) in their skeletal muscle. The study identified intramuscular protein level of β-dystroglycan as a potential biomarker of cancer cachexia. Changes in the structural elements of muscle (MyHC or dystrophin) appear to be survival biomarkers.

3.2 Introduction

In the main introduction to the thesis (Chapter 1), the concept of loss of skeletal muscle being the central characteristic of cancer cachexia was discussed. Cachexia in its advanced phase (where patients may have lost 20-30% of their body weight) is easily identified, but by this stage, it is often impossible to undertake any realistic form of multimodal rehabilitation. Thus, it might be useful to identify patients who are at risk or in the early phase of cancer cachexia so that targeted intervention can be instituted. An early intervention approach has been hampered by a limited understanding of the molecular pathways implicated in human cancer cachexia along with a lack of validated biomarkers.

Muscle wasting occurs as a result of an imbalance between protein synthesis and degradation. As discussed in Chapter 1, the majority of known signalling pathways that contribute to muscle atrophy in pre-clinical models mediate their effects through activation of the catabolic UPP (Acharyya and Guttridge, 2007, Glass, 2005). Identification of two muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1, in several distinct animal models of atrophy (Bodine et al., 2001a, Gomes et al., 2001) provides a compelling argument for a major contribution of the UPP in muscle wasting, and these are now measured commonly as surrogate indicators of UPP activation. Furthermore, insulin regulated FOXO1 and FOXO3a have been shown to increase expression of the E3 ligases MuRF1 and MAFbx in at different animal models of muscle wasting (Sandri et al., 2004, Zhao et al., 2007, Glass, 2010) highlighting these transcription factors as potential mediators of cachexia.

In humans, reduced levels of phosphorylated (inactive) FOXO3a have been observed in the skeletal muscle of cachectic compared with non-cachectic cancer patients, but a surprising twofold reduction in the amount of non-phosphorylated (active) FOXO1 and FOXO3a was also observed (Schmitt et al., 2007). In both myotube and mouse models of muscle atrophy, several autophagy genes are upregulated and FOXO3 appears to be important in inducing expression of these autophagy-related genes (Mammucari et al., 2007, Mammucari et al., 2008, Zhao et al., 2007, Zhao et al., 2008), linking the lysosomal and proteasomal systems. However, there is recent data that indicates dissociation between protein dynamics in vivo and activation or expression of the UPP-related signalling molecules in human skeletal muscle (Greenhaff et al., 2008). Thus the regulation of the UPP and the autophagy process in human cancer cachexia and their relationship to clinical status remains to be investigated fully. There is also evidence, in both pre-clinical models and some limited human data, of alterations in the dystrophin glycoprotein complex and selective degradation of myosin heavy chain (Acharyya et al., 2005, Acharyya et al., 2004, Clarke et al., 2007). Despite this knowledge, additional data relating to human cancer cachexia has been slow to appear. As discussed in Chapter 1 (Section 1.4), there are a few studies investigating potential biomarkers of cachexia in various tissue compartments using different methodological approaches. However, a comprehensive assessment in human skeletal muscle of markers known to be relevant in animal models of muscle atrophy is lacking.

Given this background and in line with the key themes of this thesis, the study described in this chapter aimed to identify potential clinically relevant biomarkers of

cachexia in skeletal muscle biopsies from UGI cancer patients in relation to weight-loss and post-operative survival. The approach taken was hypothesis-driven (Section 1.3.2) where putative candidate genes and proteins from components of pathways known to be relevant in pre-clinical models of cachexia were analysed in human skeletal muscle. Candidate markers were selected according to previous literature and included Akt and phosphorylated Akt (pAkt), FOXO transcription factors, ubiquitin E3 ligases (control of muscle anabolism/catabolism) (Acharyya et al., 2004, Lecker et al., 2004, Bodine et al., 2001a, Sandri et al., 2004, Bodine et al., 2001b), BNIP3 and GABARAPL1 (as markers of autophagy) (Lecker et al., 2004, Zhao et al., 2008, Mammucari et al., 2007, Mammucari et al., 2008), MyHC, dystrophin, β-dystroglycan and β-sarcoglycan (as markers of structural alteration in muscle) (Acharyya et al., 2004, Acharyya et al., 2005, Clarke et al., 2007, Cohen et al., 2009).

3.3 ***Hypotheses***

- Molecular changes in skeletal muscle of human cancer cachexia would mirror alterations reported in pre-clinical models of muscle atrophy
- Alterations in pathways of muscle atrophy or in the components of muscle itself would provide robust biomarkers of weight-loss and survival in human cancer cachexia

3.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

Muscle biopsy (*Rectus abdominis*) and blood collection: see Section 2.8

Blood measures (CRP): see Section 2.9

Muscle measures (Whole and nuclear protein isolation, Western blotting, antibodies, RNA isolation, qRT-PCR): see Section 2.10

Statistics: see Section 2.12.1

Additional methods

In 2009, the All Party Parliamentary Group on Cancer published a report of their inquiry into inequalities in cancer. From the evidence that the group assimilated, one of the key priority action points was to move towards reporting one-year survival rates for all types of cancer

> "to speed up early diagnosis and help sharpen the focus on measuring outcome…the advantage that such an indicator has over five-year survival rates and mortality rates is that it is concerned with events in the immediate past. It also starts to shift attention away from inputs and onto outcomes" (All Party Parliamentary Group on Cancer, 2009).

The government subsequently adopted this policy by including it as an improvement area for the NHS in the proposed outcomes framework. Thus one-year survival was used as a cut-off for subsequent analysis in this Chapter.

3.5 ***Results***

107 patients were recruited in total (15 HC and 92 UGI cancer patients). Demographics for the entire cohort are illustrated in Table 3.1. Patients were classified as cachectic if they had weight-loss ≥5%. Compared with HC, cancer patients were older (mean (SD) age 65 (10) vs. 56 (17) years, p=0.003), had higher average weight-loss (8.1 (9.3) vs. 0 (0) %, p=0.001), lower BMI (25.7 (4.0) vs. 28.0 (4.5) kg/m2, p=0.046) and a significantly lower KPS (89 (13) vs. 100 (0), p=0.001) (Table 3.1).

Within the cancer patient cohort, cachectic patients compared with non-cachectic patients had a larger proportion of females than males (19/51 vs. 7/41, Chi squared p=0.033), were younger (63 (9) vs. 68 (9) years, p=0.022), had shortened median survival (562 vs. 846 days, p=0.030), and a lower BMI (24.6 (3.7) vs. 27.0 (4.0) kg/m2, p=0.004. KPS was also significantly lower in cachectic compared with non-cachectic patients (86 (14) vs. 92 (10), p=0.020) (Table 3.1).

Analyses were performed according to availability of muscle tissue for different biomarkers. This resulted in four separate groups of patients and the demographics for these are illustrated in Table 3.2. Figure 3.1 shows the overlap between groups. No significant differences were evident between these groups and the entire cohort. Individual biomarkers were also grouped to allow optimal use of the muscle sample. For example, MyHC (molecular weight ~200 kDa) and dystrophin (~400 kDa) were analysed separately due to the requirement for 4-12% gels for Western blot and prolonged electrophoresis and transfer due to their large molecular weight.

Table 3.1: Patient demographics for the entire cohort

	Control (n=15)	**All Cancer** (n=92)	**Cancer No cachexia** (n=41)	**Cancer Cachexia** (n=51)
M/F	8/7	66/26	34/7	32/19†
Age (years)	56±17	65±10*	68±9	63±9†
Weight-loss (%)	0.0±0.0	8.1±9.3*	0.8±3.0	13.9±8.6†
Survival (days)	-	675	846	562†
BMI (kg/m^2)	28.0±4.5	25.7±4.0*	27.0±4.0	24.6±3.7†
CRP (mg/l)	3.5±2.7	15.5±31.3	12.0±29.6	18.3±32.6
CRP≥5mg/l (Y/N)	4/11	41/51	15/26	26/25
KPS	100±0	89±13*	92±10	86±14†

*Results for controls and cancer patients (with or without cachexia) are presented as mean ± SD or categorically except for median survival. * = p<0.05 cancer versus control patients, † = p<0.05 cachectic versus non-cachectic cancer patients. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score; kg, kilograms; m, metres; mg, milligrams; l, litres; Y, yes; N, no.*

Table 3.2: Individual cancer patient groups according to which biomarkers were evaluated.

	GROUP **1** (n=42)	**2** (n=52)	**3** (n=47)	**4** (n=29)
M/F	28/14	34/18	28/19	15/14
Age (years)	65±10	66±9	64±9	63±10
BMI (kg/m^2)	25.7±4.5	25.5±3.6	25.3±3.9	25.5±4.9
Weight-loss (%)	7.5±9.5	8.9±8.0	9.7±11.0	9.6±10.3
Cachexia (Y/N)	22/20	32/20	29/18	18/11
CRP (mg/l)	20.7±38.7	17.4±32.1	14.2±30.3	21.2±35.8
CRP≥5mg/l (Y/N)	20/22	26/26	21/26	23/16
KPS	83.1±13.7	92.4±10.5	91.0±11.7	83.8±13.5

Results are presented as mean ± SD. Each group was used to assess different candidate biomarkers as follows:

1= Akt, pAkt, β-dystroglycan, β-sarcoglycan

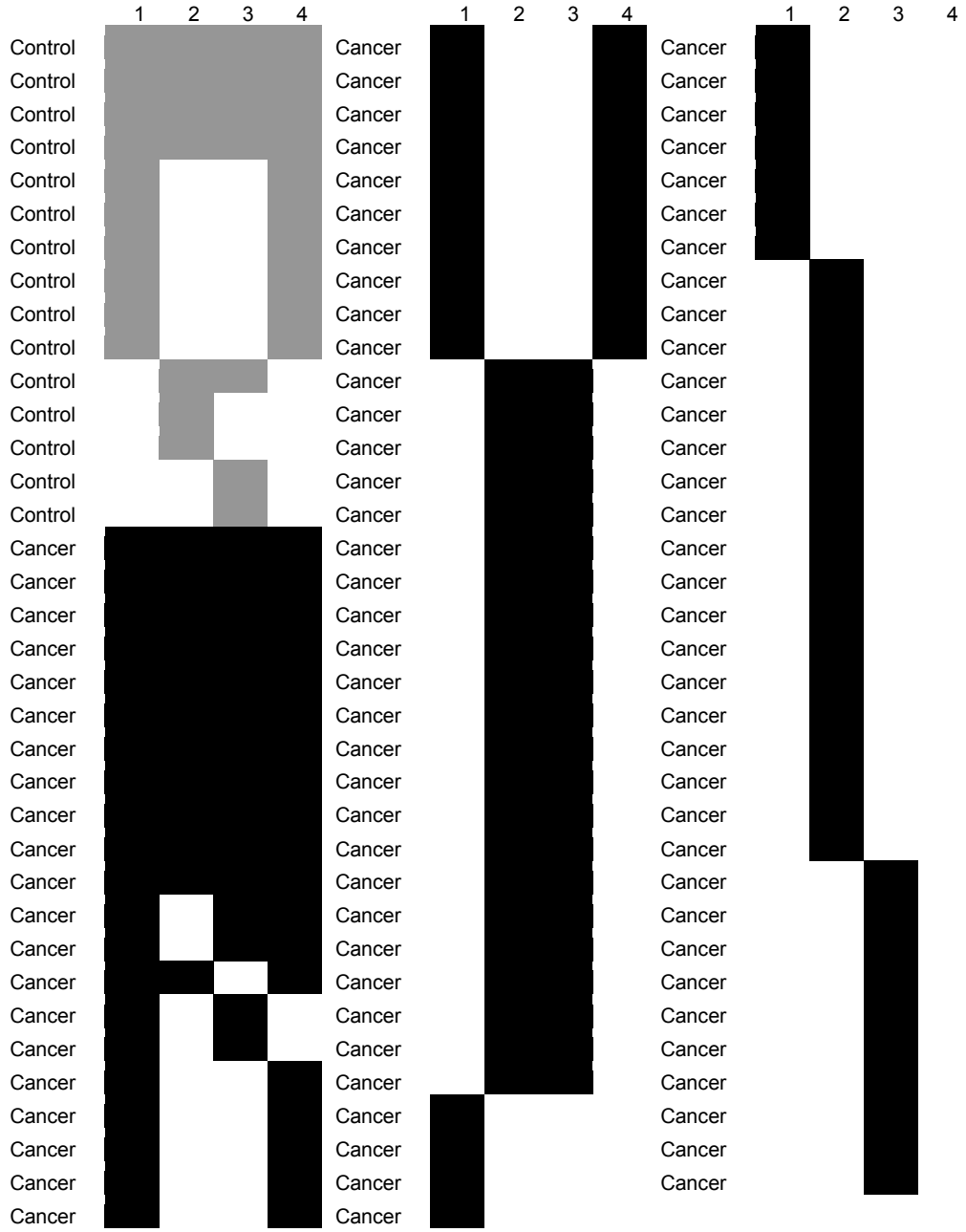
2= FOXO1, FOXO3a, BNIP3, GABARAPL1, MuRF1, MAFBx

3= MyHC

4= Dystrophin

Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score; mg, milligrams; l, litre; Y, yes; N, no.

FIGURE 3.1: VISUAL REPRESENTATION OF THE OVERLAP IN PATIENT GROUPS



All of the 107 patients recruited for this study are illustrated. Control patients are represented in grey and cancer patients in black. Only the first four controls and first eleven cancer patients had all candidate biomarkers assessed. Other patients were group specific. The groups are as per Table 3.2:

1= Akt, pAkt, β-dystroglycan, β-sarcoglycan
2= FOXO1, FOXO3a, BNIP3, GABARAPL1, MuRF1, MAFBx
3= MyHC
4= Dystrophin

Skeletal muscle protein potential biomarkers of cancer cachexia

Results of the skeletal muscle protein biomarkers of cachexia are illustrated in Figure 3.2. Level of total Akt protein was reduced in cancer patients compared with HC (0.49 (0.31) vs. 0.89 (0.17), p=0.001), but there was no significant difference in pAkt protein level (0.47 (0.34) vs. 0.29 (0.2), p=0.104). However, the ratio of pAkt to total Akt was increased in cancer patients compared with HC (1.33 (1.04) vs. 0.32 (0.21), p=0.002).

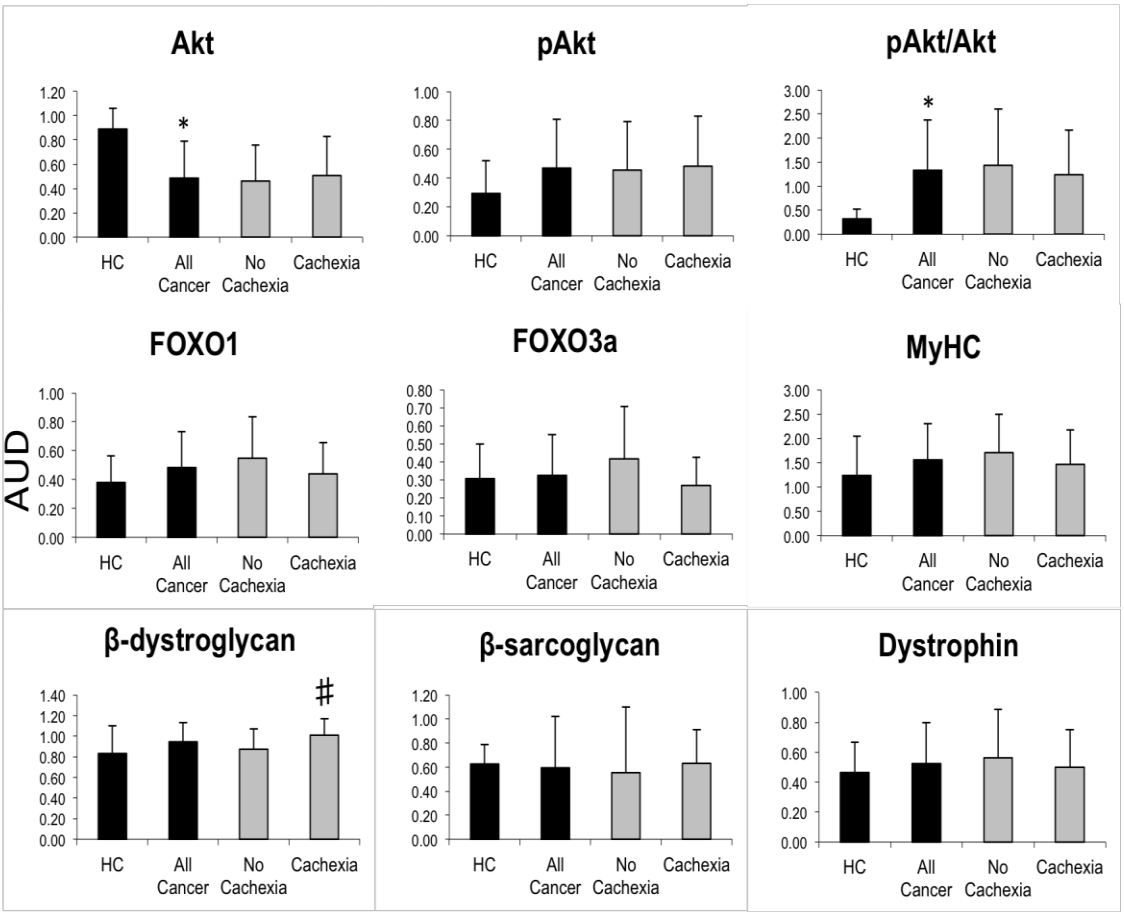
Cachectic cancer patients had significantly higher levels of β-dystroglycan than non-cachectic cancer patients (1.01 (0.16) vs. 0.87 (0.20), p=0.007). There was also a trend towards increased levels of β-sarcoglycan (0.63 (0.28) vs. 0.55 (0.55), p=0.052).

Other protein biomarkers were not significantly different in either HC vs. cancer (FOXO1, FOXO3a, MyHC, β-dystroglycan, β-sarcoglycan, dystrophin) or in cachectic compared with non-cachectic cancer patients (Akt, pAkt, pAkt/Akt ratio, FOXO1, FOXO3a, MyHC, dystrophin) (Figure 3.2).

Skeletal muscle mRNA potential biomarkers of cancer cachexia

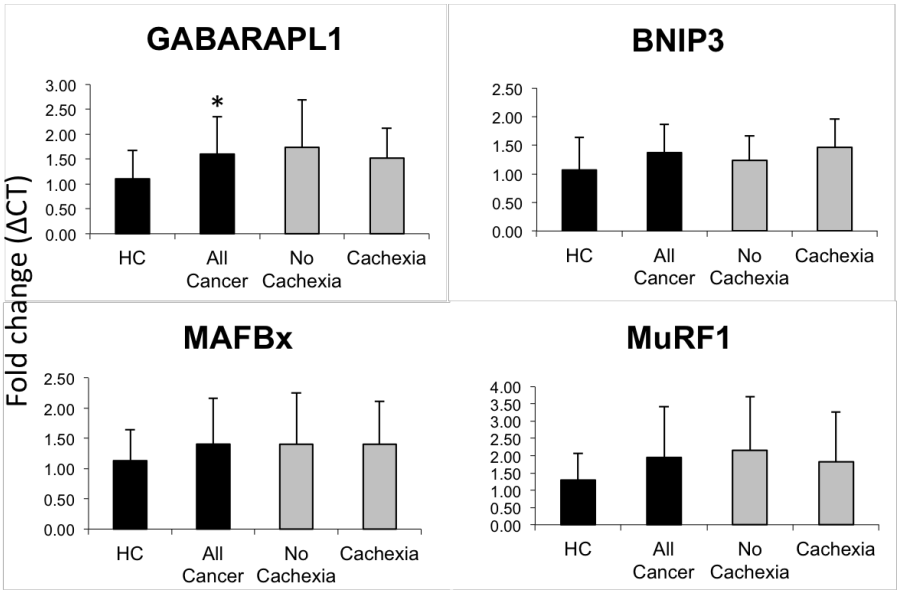
Results of the skeletal muscle mRNA biomarkers of cachexia are illustrated in Figure 3.3. There was a significantly increased expression of GABARAPL1 (1.60 (0.76) vs. 1.10 (0.57), p=0.024) and a trend towards an increase in expression of BNIP3 in cancer patients compared with HC (1.37 (0.49) vs. 1.07 (0.57), p=0.058). MuRF1 and MAFBx expression levels were not significantly different in cancer patients

FIGURE 3.2: DENSITOMETRY LEVELS FOR PROTEIN BIOMARKERS



*Graphs showing levels (AUD) of protein biomarkers in HC and cancer patients (with or without cachexia) normalised to loading control. *p<0.05 HC vs. all cancer, #p<0.05 no cachexia vs. cachexia. Abbreviations: AUD, arbitrary units of densitometry; HC, healthy controls.*

FIGURE 3.3: FOLD CHANGE IN MRNA BIOMARKERS



*Graphs showing fold change (ΔCT) of mRNA biomarkers in HC and cancer patients (with or without cachexia). *p<0.05 HC vs. all cancer. Abbreviations: HC, healthy controls; ΔCT, delta (delta) cycle threshold.*

compared with HC. None of the mRNA potential biomarkers related to the presence of cachexia.

Skeletal muscle potential biomarkers and survival

Patients who survived ≤1 year post-operatively compared with those who survived >1 year had significantly higher average weight-loss (12.0 (11.1) vs. 6.3 (8.0) %, p=0.007) and a lower KPS (83 (13) vs. 91 (12), p=0.004) (Table 3.3).

Given that there are no 'normal' cut-offs for skeletal muscle protein levels or mRNA expression of potential markers, Receiver Operating Characteristic (ROC) analysis was performed for all of the selected markers. Although there were no strong significant candidates, there was a trend for MyHC, dystrophin and pAkt (area under the curves were 0.674 (p=0.069), 0.714 (p=0.070) and 0.669 (p=0.068) respectively). Co-ordinates of the ROC curves were inspected and a cut-off of ≥0.87 AUD chosen for MyHC to give a sensitivity of 84.8% and specificity of 46.2%; a cut-off of ≥0.31 AUD chosen for dystrophin to give a sensitivity of 85.0% and specificity of 55.6%; and a cut-off of ≥0.19 AUD chosen for pAkt to give a sensitivity of 87.5% and specificity of 42.3%. Kaplan-Meier survival analysis using these cut-offs (Figure 3.4) showed a significantly shorter survival for those with lower compared with higher MyHC levels (median survival 316 vs. 1326 days, p=0.023) and lower compared with higher dystrophin levels (median survival 341 vs. 660 days, p=0.008), but no significant difference between high and low pAkt levels (p=0.320).

To examine further the difference in survival for MyHC and dystrophin, the demographics of the low versus higher levels of MyHC and dystrophin groups were inspected (Table 3.4). None of the variables differed significantly between the groups for either MyHC or dystrophin.

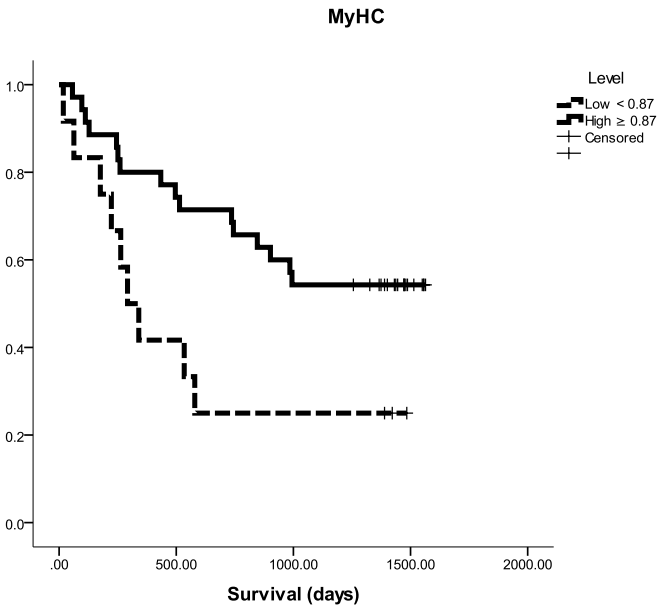
Table 3.3: Patient demographics for cancer patients surviving ≤ 1 and > 1 year.

	Survival ≤1yr (n=27)	**Survival>1yr** (n=64)
M/F	18/9	48/16
Age (years)	66±10	65±10
Weight-loss (%)	12.0±11.1	6.3±8.0*
Survival (days)	245	1195*
BMI (kg/m^2)	25.3±3.5	26.0±4.2
CRP (mg/l)	18.0±36.3	14.6±29.4
CRP≥5mg/l (Y/N)	16/11	25/39
KPS	83±13	91±12*

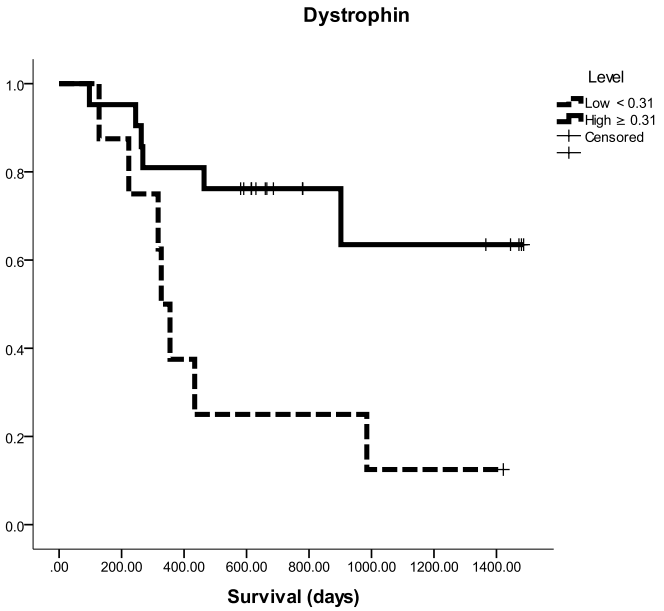
*Results are presented as mean ± SD or categorically except for median survival. * = p<0.05 survival >1yr vs survival ≤1yr. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score; kg, kilograms; m, metres; mg, milligrams; l, litres; Y, yes; N, no.*

FIGURE 3.4: SURVIVAL CURVES FOR MYHC AND DYSTROPHIN

A.



B.



*Kaplan-Meier survival analysis was done comparing groups according to ROC derived AUD cut-offs curves for (**A**) low (<0.87 AUD) vs high (≥0.87 AUD) MyHC protein levels; Log Rank p=0.023 and (**B**) low (<0.31 AUD) vs high (≥0.31 AUD) dystrophin protein levels; Log Rank p=0.008. Abbreviations: ROC, receiver operating characteristic; AUD, arbitrary units of densitometry.*

Table 3.4: Patient demographics according to MyHC and Dystrophin protein levels

A. MyHC

	Low (n=12)	**High** (n=35)
M/F	7/5	21/14
Age (years)	63±7	64±10
BMI (kg/m^2)	24.9±4.4	25.4±3.8
Weight-loss (%)	9.8±7.4	9.6±12.1
Cachexia (Y/N)	9/3	20/15
CRP (mg/l)	20.3±41.6	12.1±25.8
CRP≥5mg/l (Y/N)	7/5	14/21
KPS	88.2±10.8	92.1±12.1

B. Dystrophin

	Low (n=8)	**High** (n=21)
M/F	5/3	10/11
Age (years)	62±13	63±9
BMI (kg/m^2)	26.8±6.3	25.0±4.3
Weight-loss (%)	9.5±7.5	9.7±11.3
Cachexia (Y/N)	7/5	14/21
CRP (mg/l)	45.8±52.3	11.8±22.4
CRP≥5mg/l (Y/N)	5/3	8/13
KPS	82.5±8.9	84.3±15.0

*Results are presented as mean ± SD or categorically. ROC analysis was used to determine AUD cut-offs for (**A**) MyHC (low<0.87 AUD, high ≥0.87 AUD) and (**B**) Dystrophin (low <0.31 AUD, high ≥0.31AUD). No significant differences were found between groups. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score; ROC, receiver operating characteristic; kg, kilograms; m, metres; mg, milligrams; l, litres; Y, yes; N, no; AUD, arbitrary units of densitometry.*

3.6 ***Discussion***

Contrary to the initial hypothesis, very few of the candidate markers of muscle atrophy derived from pre-clinical models mirrored changes in human skeletal muscle. Only increased β-dystroglycan appeared to relate to the presence of significant weight-loss in cancer patients. There appeared to be suppression of total Akt protein levels in the skeletal muscle of cancer patients but with a relative increase in Akt activity (expressed as the ratio of phosphorylated to total Akt). There was also some evidence of increased autophagy (increased GABARAPL1) in cancer patients. Low MyHC and low dystrophin protein levels both related to shortened survival.

In one of very few similar studies, Schmitt et al examined protein levels and phosphorylation status of muscle atrophy/hypertrophy pathway components in eight pancreatic cancer patients with cachexia compared with eight weight-stable cancer or pancreatitis patients (Schmitt et al., 2007). Reduced levels of Akt, MyHC and FOXO1 were observed in the cachectic group. In the current study, which looked at a much larger cohort of patients with a variety of UGI cancers, along with non-cancer HC, we did not observe any differences in these markers between cachectic and non-cachectic patients. Although cancer patients (compared with non-cancer HC) did have a reduction in Akt levels, there was a relative increase in overall Akt activity (expressed as the ratio of pAkt to total Akt). It should be noted that whilst FOXO3 antibody and methodology for determining MyHC levels differed, the antibodies for FOXO1, Akt and pAkt were the same between the present study and that by Schmitt and coworkers and would thus not explain these contrasting results. An alternative explanation is that the observations of Schmitt et al may be tumour

specific or reflect markers of more moderate cachexia whereas our observations relate to the presence of cancer alone or to cachexia earlier in the disease process. Schmitt et al defined cachexia as >10% weight-loss in 6 months whereas for the current study, cachexia was defined as ≥5% weight-loss in line with recent definitions (Fearon et al., 2011). However, when analysed according to a 10% weight-loss cut-off, the results for individual variables from the current study did not differ from using a 5% weight-loss cut-off. Another explanation for the Akt results in the present study would be that total protein turnover might be suppressed in cancer patients with a reduction in the available pool of Akt and that increased phosphorylation represents a compensatory mechanism. In support of this, in COPD patients with cachexia, an increased ratio of pAkt to total Akt has also been observed with the suggestion that this represents an attempt to restore muscle mass (Doucet et al., 2007, Vogiatzis et al., 2010).

After the discovery that they were upregulated in several distinct models of atrophy (Lecker et al., 2004), the muscle-specific E3 ubiquitin ligases, MuRF-1 and atrogin-1/MAFbx have been used commonly to indicate activation of the UPP. Likewise, markers of autophagy have been shown to be increased in cachectic mice and under the control of FOXO3 (Mammucari et al., 2007). However, in the current study, FOXO transcription factors and the ubiquitin E3 ligases were similar between HC and cancer patients and were not influenced by the presence of cachexia. Cancer patients did have increased expression of GABARAPL1 and a trend towards increased expression of BNIP3, both of which play a key role in autophagy. However, neither of these autophagy markers related significantly to survival or

weight-loss. There was evidence of increased β-dystroglycan protein levels in cachectic patients and a trend towards increased protein levels of β-sarcoglycan. Dysregulation of the dystrophin glycoprotein complex (DGC) is a feature of muscular dystrophies and has been associated with cachexia (Acharyya et al., 2005). In the context of muscular dystrophy there would be downregulation of all components of the DGC, but this finding has not been replicated in cancer cachexia (Acharyya et al., 2005). However, neither has an increase in protein levels been demonstrated making it difficult to interpret the findings of the current study. In a mouse model of muscular dystrophy treated with an AMPK activator, increased utrophin coincided with an increase in β-dystroglycan and resultant strengthening of the sarcolemma (Ljubicic et al., 2011). It is therefore conceivable that the relationship between β-dystroglycan and cachexia seen in the current study represents an attempt at muscle membrane repair as it enters a more dysregulated state with progressive weight-loss. Given that the current study is not mechanistic and utrophin was not investigated as a potential marker, this suggestion is speculative. Nevertheless, β-dystroglycan remains a candidate biomarker for cancer cachexia and is worthy of further evaluation.

It is striking that there was an association between low levels of structural muscle proteins and shortened survival. The lack of significant demographic differences between the low and higher level MyHC/dystrophin groups suggests that they are bona fide independent biomarkers of post-operative survival. Perhaps lower levels of these structural proteins identify a susceptible population where muscle structure/membrane integrity has already started to become compromised. In an

ideal world it would have been highly interesting to see if these patients had gone on to develop an accelerated form of cachexia. However, the difficulties of such longitudinal analyses are explored further in Chapters 5 and 9. Alterations in membrane structure and integrity have been demonstrated in colon-26 tumour bearing mice which is thought to be due, at least in part, to disruption of the DGC (Acharyya et al., 2005). This normally provides a strong mechanical link between the intracellular cytoskeleton and extracellular matrix (Lapidos et al., 2004). Mutations in the DGC cause muscular dystrophies/ cardiomyopathy, and a link with human cancer cachexia has been made. DGC deregulation was demonstrated in gastro-oesophageal cancer patients and related to the presence of significant weight-loss (>10%) and systemic inflammation, and to a shortened survival (Acharyya et al., 2005).

It has been proposed that there is selective targeting of myofibrillar proteins, in particular MyHC, in cancer cachexia (Acharyya et al., 2004, Clarke et al., 2007). In addition, myofibrillar degradation appears to occur in a time dependent manner. One animal study of muscle atrophy after denervation or fasting, demonstrated early targeting of thin filament components with subsequent loss of MyHC (Cohen et al., 2009). Adding to the concept that membrane damage is important in the pathogenesis of cancer cachexia, Skipworth and co-workers have recently shown that the presence of various myosin species in the urine of patients with oesophago-gastric cancer relates to significant (>10%) weight-loss) (Skipworth et al., 2010b). The observations in the current study that patients with lower skeletal muscle dystrophin or MyHC protein levels are associated with a shortened survival are

entirely consistent with this concept. Therefore, measurements of these structural elements in skeletal muscle may be suitable biomarkers relating to survival in UGI cancer patients. Further study is required to determine whether the mechanism of such an association relates, in part, to the subsequent development of cachexia.

By comparing cachectic, non-cachectic and HC groups, this study evaluates the potential of certain variables to act as biomarkers of cachexia. However, it is not possible to determine the precise role of these variables in cachexia by comparing the cachectic with non-cachectic groups. The patients without cachexia at diagnosis represent a mixed group some of whom will remain weight stable, but a significant other group will progress to cachexia and are therefore in a pre-cachectic state (Figure 1.12). This heterogeneity within the weight-stable group potentially masks changes in some variables that may play a role in development of cachexia/pre-cachexia. In order to further explore this area, longitudinal assessments of patients would be required to determine which individuals progress to losing weight after the initial biopsy (see Chapter 9).

It is important to appreciate that this study is capturing a snapshot in time of what is really a journey comprising early, cachectic and refractory phases (Fearon et al., 2011) in a heterogeneous population at various points on this spectrum. There is evidence that different proteolytic/synthetic pathways may be activated or repressed according to degree of weight-loss. For example, in a study of lung cancer patients with mean weight-loss of 2.9%, the lysosomal but not the UPP was activated (Jagoe et al., 2002), whereas in patients with gastric cancer and mean weight-losses of 5.2%

and 5.6% have shown increases in components of the UPP (Bossola et al., 2001, Bossola et al., 2003). There is also evidence in cancer patients (Khal et al., 2005a) that UPP activity increases with weight-loss up to 12-19% and then declines as disease severity progresses. Likewise, a recent study of gastric cancer patients showed evidence of increase in calpains in patients with minimal or no weight-loss, but did not show any difference in expression of the ubiquitin E3 ligases in cancer compared with control patients (Smith et al., 2011). Longitudinal studies in human cancer cachexia may be informative in this regard, and indeed this is one of the aims of this thesis explored in subsequent chapters (Chapters 5 and 9)

In the present study, non-cachectic patients were 5 years older than cachectic patients. Sarcopenia of ageing has been well characterised and the non-cachectic group would thus have had 5 extra years of age-related muscle wasting. It is possible that this age gap may have influenced differences in levels of biomarkers between groups. Conversely, a younger age at diagnosis of cancer is a risk factor for poorer outcome in certain tumour types (e.g. breast (Fowble et al., 1994)). Whether younger patients are more likely to suffer from cachexia or more aggressive weight-loss is not known, but represents an area for future exploration.

The potential biomarkers of cachexia in the current study were selected from evidence relating predominantly to muscle wasting in animal models. The lack of association of biomarkers with cachexia may therefore simply reflect differences between animal and human cancer cachexia. Whereas the majority of animal models of cachexia undergo rapid and profound weight-loss, human cancer cachexia is a

chronic disease process. Furthermore, in humans, there will be added confounding factors such as level of baseline physical activity, bed rest, the presence of co-morbidities, dietary preferences, personal motivation and sickness behaviour.

The majority of potential biomarkers that were evaluated relate to protein degradation rather than synthetic pathways. There is reasonable evidence to suggest that in muscle atrophy associated with ageing/ bed-rest, suppression of protein synthesis is of greater importance than increased protein degradation (Rennie et al., 2010). Therefore, it may be that studies investigating biomarkers selected from anabolic pathways may identify candidates that relate more strongly to cachexia. Alternatively, changes in muscle at the molecular level may not have a strong influence on muscle phenotype. Evidence of such dissociation has been demonstrated (Greenhaff et al., 2008) in the skeletal muscle of healthy men. Increased amino acid and insulin availability was shown to lead to changes in anabolic signalling molecules and components of the UPP, which did not result in the expected corresponding alterations in muscle protein synthesis or breakdown (Greenhaff et al., 2008). Whether this also occurs in the context of human cancer cachexia remains to be elucidated.

This Chapter has demonstrated that many of the key components of known muscle wasting pathways from preclinical models do not transpose directly to being robust biomarkers of human cachexia. β-dystroglycan is a biomarker of weight-loss in cancer patients. Skeletal muscle Akt protein levels/phosphorylation status and GABARAPL1 expression are biomarkers relating to cancer and possibly early

cachexia. MyHC and dystrophin are biomarkers associated with survival. In the next chapter, a different approach is taken to investigate potential biomarkers of cancer cachexia using a biomarker-discovery study (see Section 1.3.2).

Chapter 4: **Using transcriptomics to identify novel biomarkers in human skeletal muscle cancer cachexia**

4.1 ***Abstract***

The results from Chapter 3 did not parallel reported molecular pathways from pre-clinical cachexia studies. In the current Chapter, a different approach was taken using transcriptomics to investigate global gene changes. RNA profiling was performed on Rectus abdominis muscle biopsies from 21 patients (3 controls and 18 with UGI cancer) using the Affymetrix U133+2 platform. Quantitative significance analysis of microarrays (SAM) identified an 82-gene signature that correlated with weight-loss. Selected genes correlating with weight-loss were validated using qRT-PCR and studied as cachexia biomarkers in Diaphragma and Vastus lateralis muscle biopsies from a second independent cohort. CAMk2β correlated positively with weight-loss in all muscle groups while CAMk2 protein levels were elevated in Rectus abdominis muscle. TIE1 was positively associated with weight-loss in both Rectus abdominis and Vastus lateralis muscle groups while others (APCDD1, EIF3I, HGS, NUDC, SKG, POLRMT, and TSC2) demonstrated tissue specific expression patterns. Promoter analysis identified binding sites for SP1, ARNT.AHR and TFAP2A over-represented in the proximal promoters of the weight-loss associated genes while FOXO binding sites were under-represented, further supporting the idea that the 82-gene signature is distinct. Thus, quantitative SAM analysis was able to discover new potential molecular intramuscular biomarkers of human cancer cachexia. The exercise activated genes, CAMk2β and TIE1, correlated positively with weight-loss across muscle groups indicating that cachexia was not simply due to inactivity. This analysis supports the findings from Chapter 3 that preclinical models do not reflect accurately the molecular characteristics of human muscle wasting.

4.2 ***Introduction***

Whilst potential biomarkers relating to early cachexia, weight-loss and survival were identified in Chapter 3, the results showed an overwhelming lack of similarity between reported markers of muscle wasting in pre-clinical models and those that were tested in human muscle. The reasons for this are unclear, but possible explanations include: human and animal cancer cachexia are two different processes, the study population is too heterogeneous to discriminate with a single biomarker, the weight-loss cut-off is too crude, sample size is small resulting in a Type II error, or that the method of biomarker assessment (Western blotting/qRT-PCR) is not sensitive enough to allow stratification of patients.

Regardless of which is correct, the question of which pathways are relevant in human cancer cachexia remains unanswered. The majority of biomarkers explored in Chapter 3 related to proteolytic pathways and it might be that markers of anabolism may be more relevant. Taking a hypothesis-driven approach in biomarker discovery has been criticised due to the following potential confounders:

- the response to any disease usually involves multiple networks and pathways rather than a single molecule
- there is variability in how cells and tissues interact with their environment with potential responses in distant cells outwith a single molecule or network
- the efficacy of a single biomarker can be negated due to population heterogeneity (Kurian et al., 2007).

Although there was a good evidence base for investigating the gene and protein biomarkers in Chapter 3, some of these pitfalls may have influenced the results. As such, rather than continuing with further hypothesis-driven targeted assessment of individual markers from these pathways, a global biomarker discovery-based approach (see Section 1.3.2) was taken to identify candidate biomarkers or patterns/ signatures which may be relevant in human cancer cachexia. Transcriptomics was chosen as the method for this study.

Transcriptomics is a method employed for the global study of gene expression at the RNA level using microarray platforms. It is one of the growing number of high throughput, multi-dimensional '-omic' technologies (e.g. genomics, metabolomics, cellomics, proteomics, physiomics and pharmacogenomics), and has gained increasing popularity as a powerful research tool because microarrays are able to survey the entire human genome on a single chip. Indeed, the original description of the 'atrogenes' in animal models of cachexia utilised transcriptomics (Lecker et al., 2004). The debate over which molecular pathways are important in cancer cachexia was discussed in Section 1.2.6.3. Overall, it remains unclear what regulates muscle mass *in vivo* nor is it clear to what extent protein degradation contributes over inhibition of protein synthesis (Emery et al., 1984a, Greenhaff et al., 2008). Given the paucity of data derived from cancer cachexia patients, and the results from the preceding Chapter, this Chapter describes the profiling of global molecular changes in the skeletal muscle of cancer patients and relates these to weight-loss and systemic inflammation.

4.3 ***Hypotheses***

- Skeletal muscle of weight losing cancer patients would have a unique transcriptomic gene signature
- The use of transcriptomics would identify novel biomarkers of human cancer cachexia that could be validated in an independent cohort

4.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

Muscle biopsy (*Rectus abdominis*) and blood collection: see Section 2.8

Blood measures (CRP): see Section 2.9

Muscle measures (Whole protein isolation, Western blotting, antibodies, RNA isolation, qRT-PCR, microarray target preparation/hybridisation): see Section 2.10

Statistics (general and microarray): see Section 2.12

Additional methods

A second cohort of patients was recruited for validation from an independent clinical centre (Karolinska Institutet, Stockholm, Sweden). This part of the study was conducted by Dr. Olav Rooyackers, Karolinska Institutet, Stockholm, Sweden. These patients were undergoing surgery for oesophageal cancer and *Vastus lateralis* muscle biopsies were taken with a Bergstrom needle and *Diaphragma* biopsies were obtained by sharp dissection when possible. Weight and self-reported change in weight over time were recorded for this cohort and thus rate of weight-loss was used in these subjects. The Regional Ethics Committee in Stockholm (Sweden) supported the study.

4.5 ***Results***

Subject characteristics

Thirty-four subjects were included in the study from two centres (Edinburgh and Stockholm). Twenty-one patients were recruited (3 controls and 18 patients with UGI cancer) from centre 1 (Edinburgh). Thirteen patients with oesophageal cancer were also recruited from centre 2 (Stockholm) as a validation cohort.

Patient demographics and anthropometric characteristics are shown in Table 4.1. Average weight-loss for centre 1 cancer patients was 8.9% (range -0.5 - 22.4%). Compared with the control group, patients in centre 1 with cancer were older, had significant weight-loss and had a lower BMI. Similarly, patients in centre 2 were older and had significant weight-loss compared with centre 1 controls. Demographics were similar between centre 1 and 2 cancer patients (Table 4.1).

Microarray analysis: novel genes associated with weight-loss in cancer (centre 1)

The initial microarray study was undertaken on *Rectus abdominis* muscle samples from centre 1 patients. Using both RMA and MAS normalized data hierarchical and k-means clustering were undertaken with data where genes with a low standard deviation were removed. No obvious pattern emerged from this analysis.

Genes varying with percentage weight-loss were then determined. In the correlation analysis, the weight stable group contained both cancer patients and healthy controls

Table 4.1: Clinical data for control subjects and cancer patients from centres 1 and 2

	Centre 1 **Control** (n=3)	Centre 1 **Cancer Patients** (n=18)	Centre 2 **Cancer Patients** (n=13)
M/F	2/1	12/6	12/1
Age (years)	45(2)	67(2)*	65(1.5)*
Weight-loss (%)	0	8.9(1.6)*	7.7(2.0)*
BMI (kg/m^2)	28.5(1.7)	24.4(0.8)*	25.5(1.2)
CRP (mg/l)	2.7(0.9)	19.7(8.1)	-
MAMC (cm)	23.8((1.7)	23.7(0.5)	-

*Results are present as mean (SEM). Centre 1 – Edinburgh, UK, Centre 2 – Stockholm, Sweden. * $p<0.05$ compared with centre 1 control. Abbreviations: BMI, body mass index; CRP, C reactive protein; MAMC, mid arm muscle circumference; M, male; F, female; kg, kilograms; m, metres; mg, milligrams; l, litres; cm, centimetres.*

in order to identify bona fide cachexia associating genes. All genes identified by the limma analysis were also present in the SAM generated list. This latter list was used for further analysis. SAM identified 73 genes with false discovery rate (FDR)<10% which co-varied positively with weight-loss and 9 genes with a FDR<10% which co-varied negatively with weight-loss (Table 4.2).

For these 82 genes identified by SAM analysis, correlation coefficients (R) were generated using Pearson's product moment correlation. Positive coefficients ranged from R= 0.82 to 0.57, $p<0.01$ and negatively correlating genes ranged from R= -0.74 to -0.65, $p<0.01$. Each relationship was visually examined by plotting the data. Cluster analysis of the 82 genes revealed visual distinction of patients with <5% reported weight-loss from those with >5% reported weight-loss (Figure 4.1).

The majority of the genes correlating with weight-loss had not been associated previously with cachexia in humans or animal models. Notably the FOXO transcription factors and E3 ligases MURF1 and MAFbx were not on the list. This Affymetrix derived weight-loss gene-signature was validated by qRT-PCR of the 9 genes with the highest correlations (APCDD1, CAMk2β, EIF3I, HGS, NUDC, POLRMT, SGK, TIE1 and TSC2) and 8 validated the microarray data (Figure 4.2 A and B).

TABLE 4.2: Genes correlating with weight-loss.

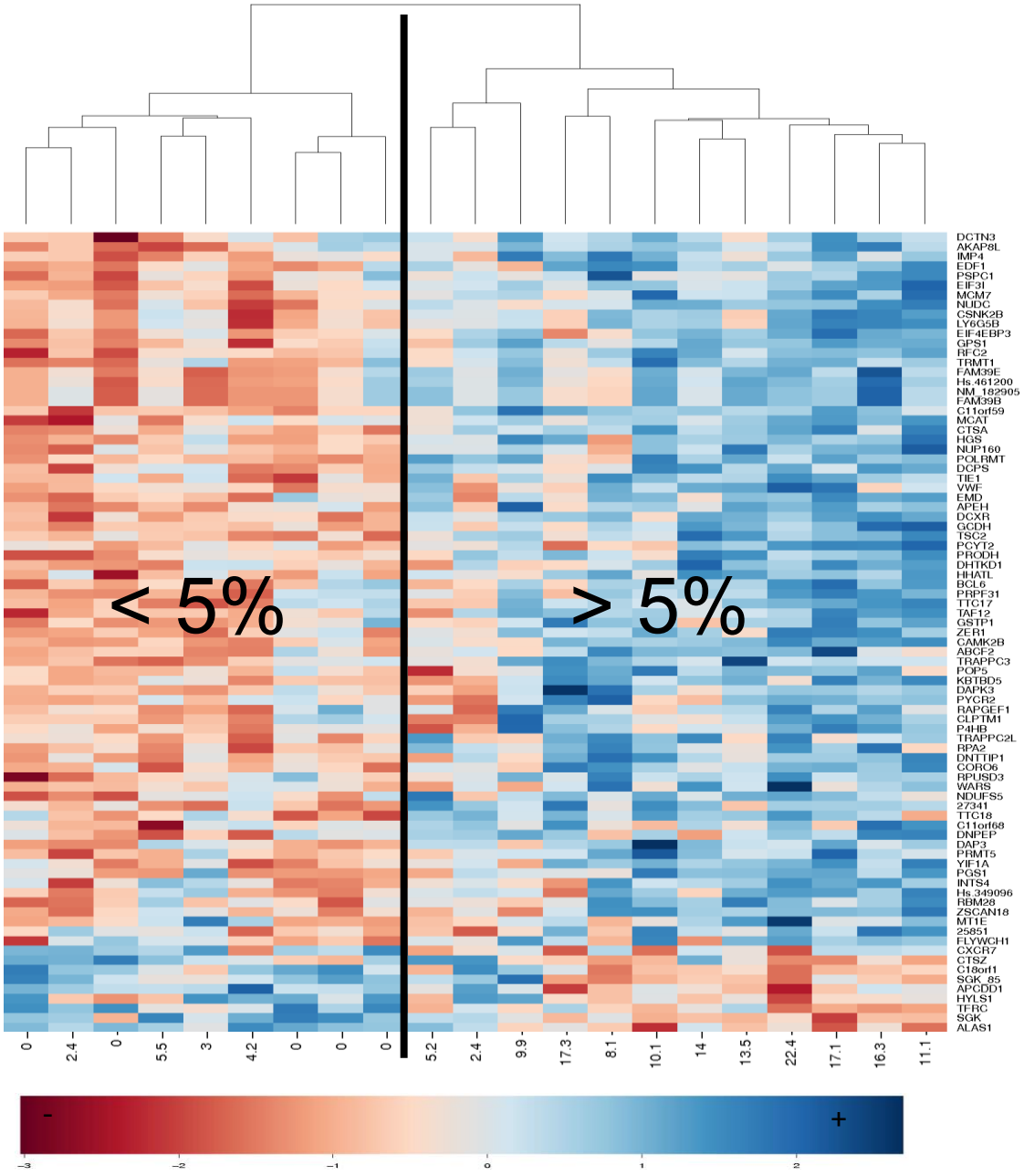
Gene	FDR (%)	R	Gene description	ENST (00000…)
CAMk2β	0	*0.82*	Calcium/calmodulin-dependent protein kinase type II beta chain	346990
ZER1	0	*0.77*	Zyg-11 related protein homolog	291900
TAF12	0	*0.74*	Transcription initiation factor TFIID subunit 12	263974
GCDH	0	*0.71*	Glutaryl-CoA dehydrogenase	222214
HGS	0	*0.70*	Hepatocyte growth factor-regulated tyrosine kinase substrate	329138
GPS1	0	*0.70*	G protein pathway suppressor 1	320548
DCTN3	0	*0.69*	Dynactin complex subunit 3	259632
TSC2	3.01	*0.69*	Tuberous sclerosis 2 protein	382538
RFC2	3.01	*0.69*	Replication factor C subunit 2	055077
NUP160	5.03	*0.68*	Nuclear pore complex protein 160	378460
APEH	3.01	*0.68*	Acylamino-acid-releasing enzyme	296456
VWF	3.01	*0.68*	von Willebrand factor precursor	261405
LY6G5B	0	*0.68*	Lymphocyte antigen 6 complex, locus G5B	375865
KBTBD5	0	*0.67*	Kelch repeat and BTB domain-containing protein 5	287777
TIE1	3.01	*0.67*	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	372475
27341	3.01	*0.66*	Gastric cancer antigen Zg14	323013
TTC18	5.03	*0.66*	Tetratricopeptide repeat protein 18	372928
PRODH	6.05	*0.66*	Proline oxidase	334029
WARS	3.01	*0.66*	Tryptophanyl-tRNA synthetase	355338
DCXR	3.01	*0.65*	Dicarbonyl/L-xylulose reductase	306869
CSNK2B	5.03	*0.65*	Casein kinase II subunit beta (CK II beta) (Phosvitin) (G5a).	383433
FLYWCH1	5.03	*0.65*	FLYWCH-type zinc finger 1 isoform a	344592
NUDC	3.47	*0.65*	Nuclear migration protein nudC (Nuclear distribution protein C homolog)	321265
TRMT1	5.03	*0.65*	N(2)	357720
PRPF31	6.05	*0.65*	U4/U6 small nuclear ribonucleoprotein Prp31 (Pre-mRNA-processing factor 31)	263436
INTS4	5.03	*0.65*	Integrator complex subunit 4 (Int4).	354849
FAM39E	5.03	*0.65*	FAM39B protein (Fragment)	359512
25851	5.03	*0.64*	0	379795
DNPEP	6.05	*0.64*	Aspartyl aminopeptidase (EC 3.4.11.21).	322176
RPUSD3	5.03	*0.64*	RNA pseudouridylate synthase domain containing 3	287624
RPA2	3.01	*0.64*	Replication protein A 32 kDa subunit	373909

EIF3I	5.03	*0.64*	Eukaryotic translation initiation factor 3 subunit 2 (eIF-3 beta) (eIF3 p36) (eIF3i)	373586
C11orf59	5.03	*0.64*	UPF0404 protein C11orf59	278671
CORO6	3.01	*0.64*	Coronin-6.	337761
EIF4EBP3	5.03	*0.64*	Eukaryotic translation initiation factor 4E-binding protein 3 (4E-BP3) (eIF4E-binding protein 3).	297183
P4HB	5.03	*0.63*	Protein disulfide-isomerase precursor (EC 5.3.4.1) (PDI) (Prolyl 4- hydroxylase subunit beta)	331483
MCAT	5.03	*0.63*	Malonyl CoA-acyl carrier protein transacylase	290429
Hs.461200	5.03	*0.63*	Protein FAM39A.	378819
YIF1A	5.03	*0.63*	Protein YIF1A (YIP1-interacting factor homolog A)	376901
PYCR2	5.03	*0.63*	Pyrroline-5-carboxylate reductase 2 (EC 1.5.1.2) (P5CR 2) (P5C reductase 2).	316940
ABCF2	5.03	*0.63*	ATP-binding cassette sub-family F member 2 (Iron-inhibited ABC transporter 2).	222388
C11orf68	6.05	*0.63*	Uncharacterized protein C11orf68 (Basophilic leukemia-expressed protein Bles03) (Protein p5326).	312536
NDUFS5	5.03	*0.63*	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	372969
TRAPPC2L	6.05	*0.62*	trafficking protein particle complex 2-like	301021
PCYT2	5.03	*0.62*	Ethanolamine-phosphate cytidylyltransferase (EC 2.7.7.14) (Phosphorylethanolamine transferase) (CTP:phosphoethanolamine cytidylyltransferase).	331285
CTSA	5.03	*0.62*	Lysosomal protective protein precursor (EC 3.4.16.5) (Cathepsin A) (Carboxypeptidase C) (Protective protein for beta-galactosidase)	191018
PRMT5	5.03	*0.62*	Protein arginine N-methyltransferase 5	324366
HHATL	5.03	*0.62*	Glycerol uptake/transporter homolog.	310417
DAP3	5.03	*0.62*	Mitochondrial 28S ribosomal protein S29 (S29mt) (MRP-S29) (Death- associated protein 3) (DAP-3)	343043
DHTKD1	5.03	*0.62*	dehydrogenase E1 and transketolase domain containing protein 1	263035
RAPGEF1	5.03	*0.61*	Rap guanine nucleotide exchange factor 1 (Guanine nucleotide-releasing factor 2)	372195
TTC17	5.03	*0.61*	Tetratricopeptide repeat protein 17	039989
DCPS	5.03	*0.61*	Scavenger mRNA-decapping enzyme DcpS	263579
IMP4	5.03	*0.61*	U3 small nucleolar ribonucleoprotein protein IMP4 (U3 snoRNP protein IMP4)	259239
MT1E	5.03	*0.60*	Metallothionein-1E (MT-1E) (Metallothionein-IE)	306061
Hs.349096	5.03	*0.60*	Small VCP/p97-interacting protein.	354193
POLRMT	5.03	*0.60*	DNA-directed RNA polymerase	215591
MCM7	5.03	*0.60*	DNA replication licensing factor MCM7 (CDC47 homolog)	362082
BCL6	5.03	*0.60*	B-cell lymphoma 6 protein (BCL-6)	232014

NM_182905	5.03	*0.60*	family with sequence similarity 39	330546
FAM39B	5.03	*0.60*	Protein FAM39B	285718
PGS1	5.03	*0.60*	phosphatidylglycerophosphate synthase 1	262764
EDF1	5.03	*0.60*	Endothelial differentiation-related factor 1 (EDF-1)	371648
AKAP8L	5.03	*0.59*	A-kinase anchor protein 8-like (AKAP8-like protein)	263378
GSTP1	5.03	*0.59*	Glutathione S-transferase P	196968
PSPC1	5.03	*0.59*	paraspeckle protein 1	335781
EMD	5.03	*0.58*	Emerin.	369842
POP5	5.03	*0.58*	Ribonuclease P/MRP protein subunit POP5	357500
TRAPPC3	5.03	*0.58*	Trafficking protein particle complex subunit 3	373159
RBM28	5.03	*0.58*	RNA-binding protein 28 (RNA-binding motif protein 28)	223073
DNTTIP1	5.03	*0.58*	Terminal deoxynucleotidyltransferase-interacting factor 1 (TdT- interacting factor 1) (TdIF1)	372622
CLPTM1	5.03	*0.57*	Cleft lip and palate transmembrane protein 1	337392
DAPK3	5.03	*0.57*	Death-associated protein kinase 3	301264
ZSCAN18	5.03	*0.57*	Zinc finger and SCAN domain-containing protein 18	240727
CXCR7	9.95	*-0.65*	C-X-C chemokine receptor type 7	272928
SGK_85	3.01	*-0.65*	Uncharacterized serine/threonine-protein kinase SgK085 (EC 2.7.11.1) (Sugen kinase 85)	274643
ALAS1	9.95	*-0.65*	5-aminolevulinate synthase	310271
CTSZ	9.95	*-0.66*	Cathepsin Z precursor (EC 3.4.22.-) (Cathepsin X) (Cathepsin P)	217131
SGK	3.47	*-0.68*	Serine/threonine-protein kinase Sgk1 (EC 2.7.11.1) (Serum/glucocorticoid-regulated kinase 1	367858
HYLS1	9.95	*-0.71*	Hydrolethalus syndrome protein 1	356438
TFRC	0	*-0.72*	Transferrin receptor protein 1	360110
APCDD1	0	*-0.74*	Protein APCDD1 precursor (Adenomatosis polyposis coli down-regulated 1 protein)	355285

Using U133+2 Affymetrix chips and MAS5/RMA normalisation, a quantitative SAM analysis was carried out. Genes that correlated positively and negatively with weight-loss were identified (FDR<10%) and correlation coefficients generated separately using regression analysis. All data shown have a correlation p-value <0.05. Genes that are highlighted in grey were selected for qRT-PCR validation. Abbreviations: FDR, false discover rate; R, Pearson's correlation coefficient; ENST, Ensemble transcript identifier.

FIGURE 4.1: CLUSTER ANALYSIS IDENTIFIES HIGH AND LOW WEIGHT-LOSS GROUPS



Transcriptomics identified an 82 gene-signature correlating with weight-loss. These genes are listed down the right-hand side. Individual patients' weight-loss is plotted along the bottom. Expression data from these genes were used to drive cluster analysis and produce a heatmap. Red represents decreased expression and blue, increased expression. Two clusters of subjects were revealed; high weight-loss (≥5%) and low weight loss (<5%).

FIGURE 4.2A: HEATMAP REPRESENTING MICROARRAY VERSUS QRT-PCR VALIDATION FOR COVARYING GENES

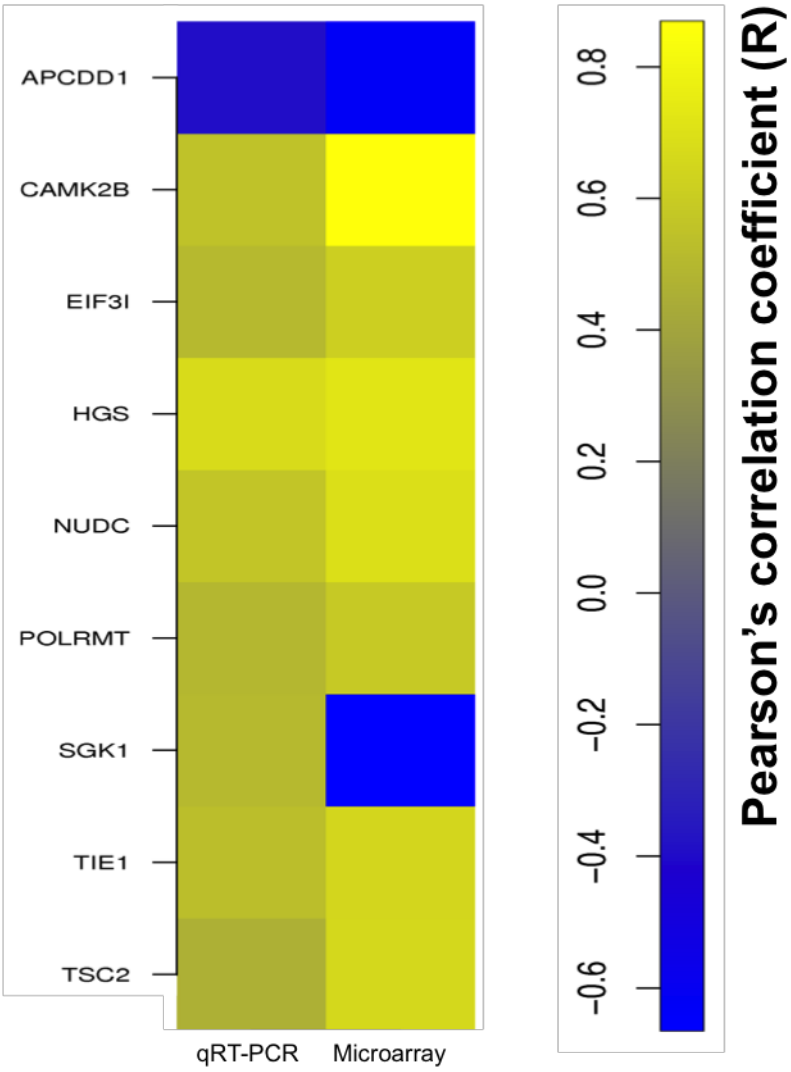
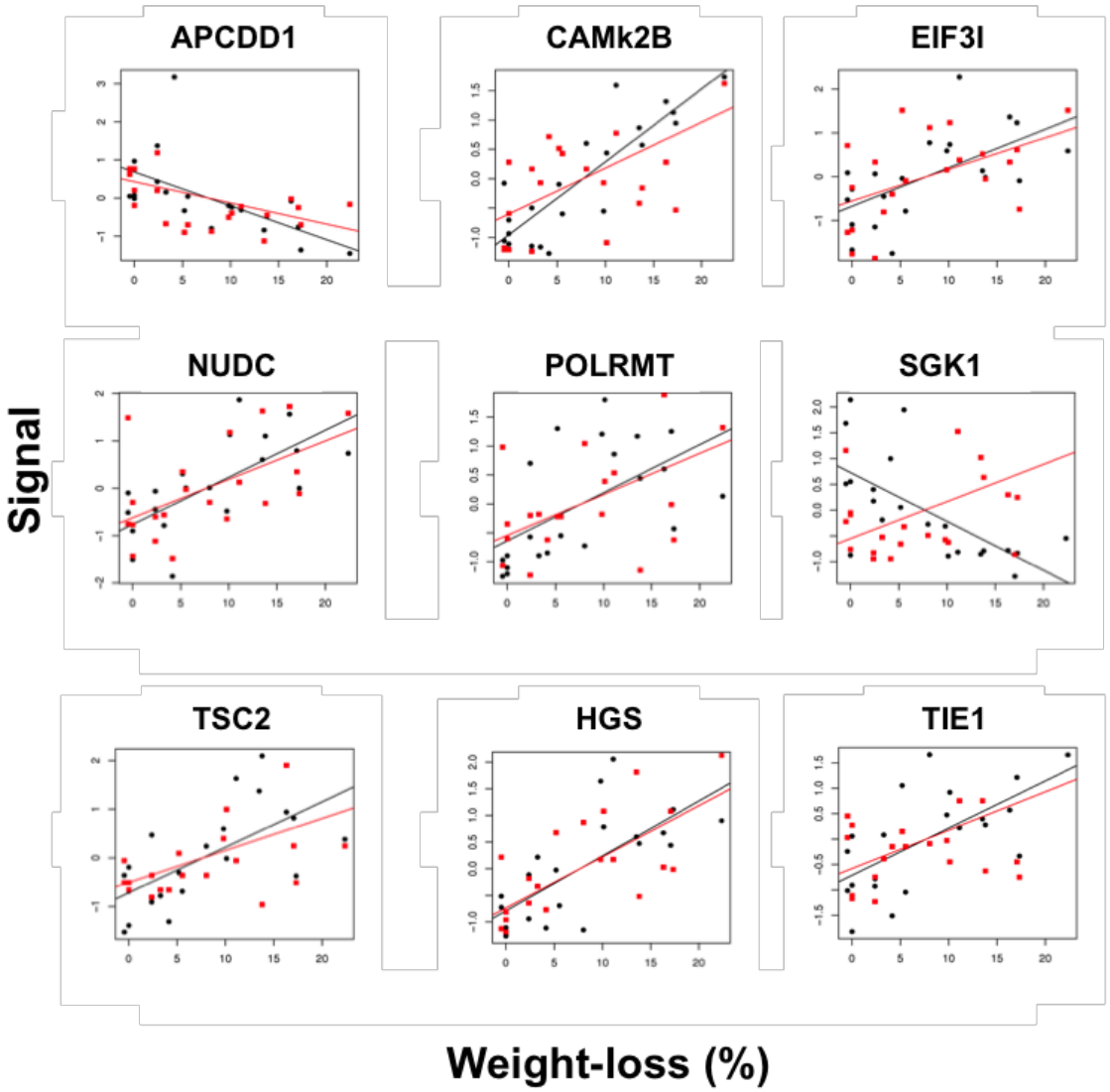


FIGURE 4.2B: CORRELATION PLOTS FOR IDENTIFIED COVARYING GENES



*From the 82 gene-signature correlating with weight-loss, 9 were selected for validation by qRT-PCR. A heatmap of Pearson's correlation coefficients (**A.**) and correlation plots (**B.**) for array data and qRT-PCR data with weight-loss are shown. The qRT-PCR and array data were scaled to make them comparable and each dataset is plotted against % weight-loss. For each dataset, the least square line is also shown. P-values for the correlations ranged from 0.03 to below 0.01. All genes except SGK1 validated the array. Red = qRT-PCR; black = microarray.*

Microarray analysis: novel genes associated with systemic inflammation in cancer (Centre 1)

Some of the key systemic mediators of cancer cachexia are cytokines and the acute phase response (see Section 1.2.6.2). In order to explore the relationship between systemic inflammation and gene changes in cancer cachexia, a provisional analysis of the microarray data was performed as described above for weight-loss but using CRP in its place. This analysis was not corrected for further multiple testing beyond that afforded by the FDR calculation in SAM. Patients without systemic inflammation (CRP <5mg/l) were also included to identify inflammation related genes rather than genes associated with cancer alone. 76 genes (FDR<10%) correlated positively with systemic inflammation and 25 genes (FDR<10%) had a significant negative correlation (Table 4.3).

Correlation coefficients ranged from R = 0.82 to 0.44 for positively correlating genes and R = -0.44 to -0.72 for negatively correlating genes ($p<0.05$). Positively correlating genes included the transcription factor FOXO1 and the autophagy related gene GABARAPL1 but not the E3 ligases MuRF1 and MAFBx. In contrast to the weight-loss gene signature (Figure 4.1), clustering with this gene-list did not easily differentiate high from low systemic inflammation subjects and no divisive gene signature could be distinguished. Given these findings, subsequent work was focused exclusively on weight-loss associated genes.

Table 4.3: Genes correlating with systemic inflammation

Gene	FDR (%)	R	Gene description	ENST (00000…)
ZNF547	8.42	*0.44*	Zinc finger protein 547	282282
C18orf21	5.91	*0.45*	Uncharacterized protein C18orf21 (HBV X-transactivated gene 13 protein)	333234
C20orf111	8.42	*0.45*	Uncharacterized protein C20orf111	255174
RPP21	8.42	*0.45*	Ribonuclease P protein subunit p21	376644
FOXO1	7.06	*0.45*	Forkhead box protein O1	379561
WDR74	7.06	*0.45*	WD repeat-containing protein 74	278856
BTAF1	8.42	*0.46*	TATA-binding protein-associated factor 172	265990
UBE2E1	8.42	*0.47*	Ubiquitin-conjugating enzyme E2 E1	346855
FAM107A	8.42	*0.47*	Protein FAM107A	360997
MSH3	8.42	*0.47*	DNA mismatch repair protein Msh3 (Divergent upstream protein) (DUP)	265081
XPC	7.45	*0.47*	DNA-repair protein complementing XP-C cells (Xeroderma pigmentosum group C-complementing protein) (p125)	285021
CTNS	2.96	*0.47*	Cystinosin	046640
EIF3M	8.42	*0.48*	Eukaryotic translation initiation factor 3 subunit M	323213
GCLM	4.21	*0.48*	Glutamate--cysteine ligase regulatory subunit	370238
SLC25A27	8.42	*0.48*	Mitochondrial uncoupling protein 4 (UCP 4) (Solute carrier family 25 member 27)	355073
ASB8	5.91	*0.48*	Ankyrin repeat and SOCS box protein 8 (ASB-8)	317697
RAB3GAP2	8.42	*0.48*	Rab3 GTPase-activating protein non-catalytic subunit	358951
DAP3	5.91	*0.48*	28S ribosomal protein S29, mitochondrial (S29mt) (MRP-S29) (Death-associated protein 3)	368336
PTPN3	0.00	*0.49*	Tyrosine-protein phosphatase non-receptor type 3 (EC 3.1.3.48) (Protein-tyrosine phosphatase H1)	374541
PDSS2	7.45	*0.49*	Decaprenyl-diphosphate synthase subunit 2 (EC 2.5.1.-) (Decaprenyl pyrophosphate synthetase subunit 2)	369031
SPSB1	8.42	*0.49*	SPRY domain-containing SOCS box protein 1 (SSB-1)	357898
ADFP	8.42	*0.49*	Adipophilin (Adipose differentiation-related protein) (ADRP)	276914
FAM134B	5.91	*0.50*	Protein FAM134B	306320
RAD50	8.42	*0.50*	DNA repair protein RAD50 (EC 3.6.-.-) (hRAD50)	265335
PSMG4	8.42	*0.50*	Proteasome assembly chaperone 4 (PAC-4) (hPAC4)	380303
CCDC86	8.42	*0.50*	Coiled-coil domain-containing protein 86	227520
GTF2A2	8.42	*0.50*	Transcription initiation factor IIA subunit 2 (General transcription factor IIA subunit 2)	267869
DDX21	7.45	*0.50*	Nucleolar RNA helicase 2 (EC 3.6.1.-) (Nucleolar RNA helicase II) (DEAD box protein 21)	354185
FAM120B	8.42	*0.51*	Constitutive coactivator of peroxisome proliferator-activated receptor gamma	366751
CREM	8.42	*0.51*	cAMP-responsive element modulator (Inducible cAMP early repressor) (ICER)	374734
CENPQ	8.42	*0.51*	Centromere protein Q (CENP-Q)	371200
HMGB2	7.45	*0.52*	High mobility group protein B2 (High mobility group protein 2) (HMG-2)	296503
TXNL1	8.42	*0.52*	Thioredoxin-like protein 1	217515

REPS1	8.42	*0.52*	RalBP1-associated Eps domain-containing protein 1	258062
PRMT5	7.06	*0.53*	Protein arginine N-methyltransferase 5	324366
GNPAT	8.42	*0.53*	Dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42) (DHAP-AT) (DAP-AT) (Glycerone-phosphate O-acyltransferase)	366646
CNOT10	8.42	*0.53*	CCR4-NOT transcription complex subunit 10	328834
TIMM9	8.42	*0.54*	Mitochondrial import inner membrane translocase subunit Tim9	216463
RASL10B	8.42	*0.54*	Ras-like protein family member 10B Precursor	268864
MAP3K2	7.45	*0.54*	Mitogen-activated protein kinase kinase kinase 2	344908
TMEM111	5.42	*0.54*	Transmembrane protein 111	245046
DCP1A	7.45	*0.54*	mRNA-decapping enzyme 1A (EC 3.-.-.-) (Transcription factor SMIF) (Smad4-interacting transcriptional co-activator)	294241
ATAD2	8.42	*0.54*	ATPase family AAA domain-containing protein 2	287394
ZNF197	7.45	*0.54*	Zinc finger protein 197 (ZnF20) (Zinc finger protein with KRAB and SCAN domains 9)	344387
CIRH1A	8.42	*0.55*	Cirhin	352319
EIF2A	7.45	*0.55*	Eukaryotic translation initiation factor 2A	383043
MNAT1	7.45	*0.55*	CDK-activating kinase assembly factor MAT1 (RING finger protein MAT1) (Menage a trois)	261245
JMJD2C	0.00	*0.55*	JmjC domain-containing histone demethylation protein 3C (EC 1.14.11.-) (Jumonji domain-containing protein 2C)	381306
NFIL3	5.91	*0.56*	Nuclear factor interleukin-3-regulated protein (E4 promoter binding-protein 4)	297689
EIF3E	8.42	*0.56*	Eukaryotic translation initiation factor 3 subunit E	220849
ITSN2	5.91	*0.56*	Intersectin-2 (SH3 domain-containing protein 1B)	355123
DPY30	5.42	*0.56*	Protein dpy-30 homolog (Dpy-30-like protein)	342166
EIF2B2	8.42	*0.57*	Translation initiation factor eIF-2B subunit beta	266126
PTCH1	8.42	*0.57*	Protein patched homolog 1 (PTC1) (PTC)	375284
C13orf27	8.42	*0.57*	Uncharacterized protein C13orf27	376019
SLBP	8.42	*0.58*	Histone RNA hairpin-binding protein (Histone stem-loop-binding protein)	318386
MINA	7.45	*0.58*	MYC-induced nuclear antigen (Mineral dust-induced gene protein)	333396
ZNF302	8.42	*0.58*	Zinc finger protein 302 (ZNF135-like) (ZNF140-like)	221282
HEBP1	5.91	*0.59*	Heme-binding protein 1 (p22HBP)	014930
LPIN1	8.42	*0.59*	Lipin-1	256720
DYNC2LI1	7.45	*0.60*	Cytoplasmic dynein 2 light intermediate chain 1 (Dynein 2 light intermediate chain)	260605
MPHOSPH10	8.42	*0.61*	U3 small nucleolar ribonucleoprotein protein MPP10 (M phase phosphoprotein 10)	244230
UNC50	8.42	*0.62*	Protein unc-50 homolog (Uncoordinated-like protein)	357765
CYP4B1	8.42	*0.62*	Cytochrome P450 4B1	271153
MCM7	7.45	*0.62*	DNA replication licensing factor MCM7 (CDC47 homolog)	343023
GABARAPL1	8.42	*0.63*	Gamma-aminobutyric acid receptor-associated protein-like 1	266458
UHRF1BP1	7.45	*0.64*	UHRF1-binding protein 1 (Ubiquitin-like containing PHD and RING finger domains 1-binding protein 1)	192788
OSBPL3	0.00	*0.65*	Oxysterol-binding protein-related protein 3	313367
EPB41L5	8.42	*0.65*	Band 4.1-like protein 5	263713

GPR125	8.42	*0.67*	Probable G-protein coupled receptor 125 Precursor	282943
PSMG1	7.06	*0.68*	Proteasome assembly chaperone 1 (PAC-1)	331573
HSPA14	8.42	*0.68*	Heat shock 70 kDa protein 14	378348
TMTC1	8.42	*0.71*	Transmembrane and TPR repeat-containing protein 1	256062
MTMR10	8.42	*0.71*	Myotubularin-related protein 10	340566
PHIP	8.42	*0.75*	PH-interacting protein (PHIP)	275034
HMOX1	0.00	*0.75*	Heme oxygenase 1	216117
USP13	8.42	*0.82*	Ubiquitin carboxyl-terminal hydrolase 13 (Ubiquitin-specific-processing protease 13)	263966
CXCL12	5.91	*-0.72*	Stromal cell-derived factor 1 (SDF-1) (C-X-C motif chemokine 12)	343575
C5orf13	4.85	*-0.63*	Neuronal protein 3.1 (Protein p311)	257435
CASQ1	5.42	*-0.62*	Calsequestrin-1 Precursor	368078
EEF1A2	4.85	*-0.62*	Elongation factor 1-alpha 2 (EF-1-alpha-2)	217182
TCEAL8	7.06	*-0.61*	Transcription elongation factor A protein-like 8	360000
ANK1	0.00	*-0.61*	Ankyrin-1 (Erythrocyte ankyrin)	265709
TPM1	4.85	*-0.61*	Tropomyosin alpha-1 chain	288398
SGCD	5.91	*-0.59*	Delta-sarcoglycan (SG-delta)	337851
ANK2	7.45	*-0.58*	Ankyrin-2 (Brain ankyrin) (Ankyrin-B)	264366
PPP1R3C	0.00	*-0.58*	Protein phosphatase 1 regulatory subunit 3C	238994
ICA1	4.85	*-0.57*	Islet cell autoantigen 1	317367
SESTD1	7.45	*-0.56*	SEC14 domain and spectrin repeat-containing protein 1	335289
ALAS1	4.85	*-0.54*	5-aminolevulinate synthase, nonspecific, mitochondrial Precursor	310271
PLCD3	5.42	*-0.53*	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-3	322765
EHD4	5.42	*-0.53*	EH domain-containing protein 4	220325
IFIT1	4.85	*-0.51*	Interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1)	371804
ADAM19	0.00	*-0.50*	ADAM 19 Precursor (EC 3.4.24.-) (A disintegrin and metalloproteinase domain 19)	257527
MYBPC2	7.45	*-0.49*	Myosin-binding protein C, fast-type	357701
MEF2C	0.00	*-0.47*	Myocyte-specific enhancer factor 2C	340208
NDRG3	5.42	*-0.47*	Protein NDRG3	349004
PRRC1	7.45	*-0.46*	Protein PPRC1	296666
PAPSS1	4.85	*-0.45*	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 1	265174
SYPL2	5.42	*-0.45*	Synaptophysin-like protein 2	369872
PKM2	5.42	*-0.44*	Pyruvate kinase isozymes M1/M2	319622
LIMCH1	4.85	*-0.44*	LIM and calponin homology domains-containing protein 1	313875
ZMYND17	7.06	*-0.43*	Zinc finger MYND domain-containing protein 17	299432

Using U133+2 Affymetrix chips and MAS5/RMA normalisation, a quantitative SAM analysis was carried out. Genes that correlated positively and negatively with systemic inflammation were identified (FDR<10%) and correlation coefficients generated separately using regression analysis. All data shown have a correlation p-value <0.05. Abbreviations: FDR, false discover rate; R, Pearson's correlation coefficient; ENST, Ensemble transcript identifier.

Confirmation of genes associated with weight-loss in cancer cachexia (centre 2)

To provide biological validation of the weight-loss gene-signature generated from the centre 1 cohort, the 9 technically validated genes (APCDD1, CAMk2β, EIF3I, HGS, NUDC, POLRMT, SGK, TIE1 and TSC2) were examined using qRT-PCR in two additional types of skeletal muscle. This validation was carried out at the Karolinska Institutet, Stockholm, Sweden under the supervision of Dr O Rooyackers/Prof JA Timmons. Table 4.4 shows the results. The significant association between CAMk2β and weight-loss observed in the *Rectus abdominis* biopsies from centre 1 ($R = 0.82$; $p = 0.01$) (Figure 4.2B) was reflected by a trend in both *Vastus lateralis* biopsies ($R = 0.45$; $p = 0.06$) and *Diaphragma* biopsies ($R = 0.5$; $p = 0.07$) (Figure 4.3) from centre 2 patients (where statistical significance is partly limited by power, due to a lower sample size). TIE1 was also significantly correlated with weight-loss in both *Rectus abdominis* ($R = 0.67$; $p = 0.01$) (Figure 4.2B) and *Vastus lateralis* ($R = 0.7$; $p = 0.01$) (Figure 4.3).

CAMk2β protein and phosphorylation (Centre 1)

Given the changes observed in CAMk2β mRNA in centres 1 and 2, the protein and phosphorylation level of this kinase in the *Rectus abdominis* muscle from a larger group recruited from centre 1 (n=52) was evaluated. Table 4.5 shows the demographics of this cohort.

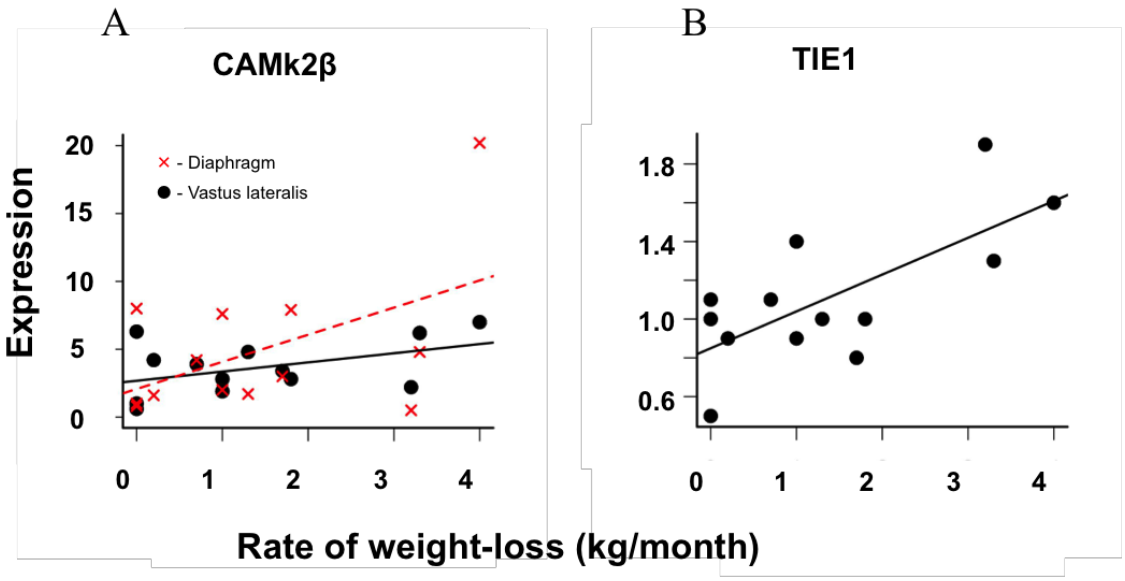
Western blotting revealed similar protein levels of CAMk2 (Figure 4.4) in weight losing and weight stable groups ($p=0.302$). However, there was a significant increase in phosphorylated (p)CAMk2 (the constitutively active form) in the muscle

Table 4.4: Validation of selected genes correlating with weight-loss (centres 1 and 2)

	Centre 1 (n=21) *Rectus abdominis*			**Centre 2** (n=13) *Vastus lateralis*		*Diaphragma*	
Gene	R		p	R	p	R	p
	Microarray	*qRT-PCR*		*qRT-PCR*		*qRT-PCR*	
APCDD1	-0.7	-0.5	0.03	0.3	0.40	-0.2	0.50
CAMk2β	0.8	0.5	0.01	0.5	0.06	0.5	0.07
EIF3I	0.6	0.5	0.02	0.1	0.70	0.2	0.40
HGS	0.7	0.7	<0.01	0.2	0.60	0.2	0.40
NUDC	0.7	0.7	<0.01	0.1	0.70	0	0.90
POLRMT	0.6	0.5	0.02	0.1	0.80	0	0.90
TIE1	0.7	0.5	0.01	0.7	0.01	0	0.90
TSC2	0.7	0.5	0.03	0.4	0.20	0	0.80

The 8 genes from the transcriptomic gene signature validated by qRT-PCR (Figure 4.2) were also examined in the cohort from Centre 2 using qRT-PCR. For each gene the correlation coefficient from the Affymetrix data set is shown followed by the correlation coefficient for qRT-PCR and a p-value for this latter regression. Abbreviations: R, Pearson's correlation coefficient.

FIGURE 4.3: CAMK2B AND TIE1 CORRELATE WITH WEIGHT-LOSS IN CANCER PATIENTS



In order to validate the findings from the Rectus abdominis, qRT-PCR was used to examine mRNA expression of CAMk2β and TIE 1 in Diaphragma (red crosses) and Vastus lateralis (closed circles) in centre 2 patients. The correlation coefficient for TIE1 in Diaphragma was not significant (see Table 4.4) and is thus not illustrated. Correlation plots for mRNA expression level against rate of weight-loss are shown. Correlation coefficients were significant with $p<0.05$.

Table 4.5: Clinical data for cohort of cancer patients from centre 1 undergoing CAMk2 Western blotting

	Centre 1 **Cancer patients** (n=52)
M/F	34/18
Age (years)	66 (1.3)
Weight-loss (%)	8.9 (1.1)
BMI (kg/m^2)	25.5 (0.5)
CRP (mg/l)	17.4 (4.4)
MAMC (cm)	24.4 (0.4)

*Results are presented as mean (SEM). Centre 1 – Edinburgh, UK. * p<0.05 compared with centre 1 control. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; MAMC, mid arm muscle circumference; kg, kilograms; m, metres; mg, milligrams; l, litres; cm, centimetres.*

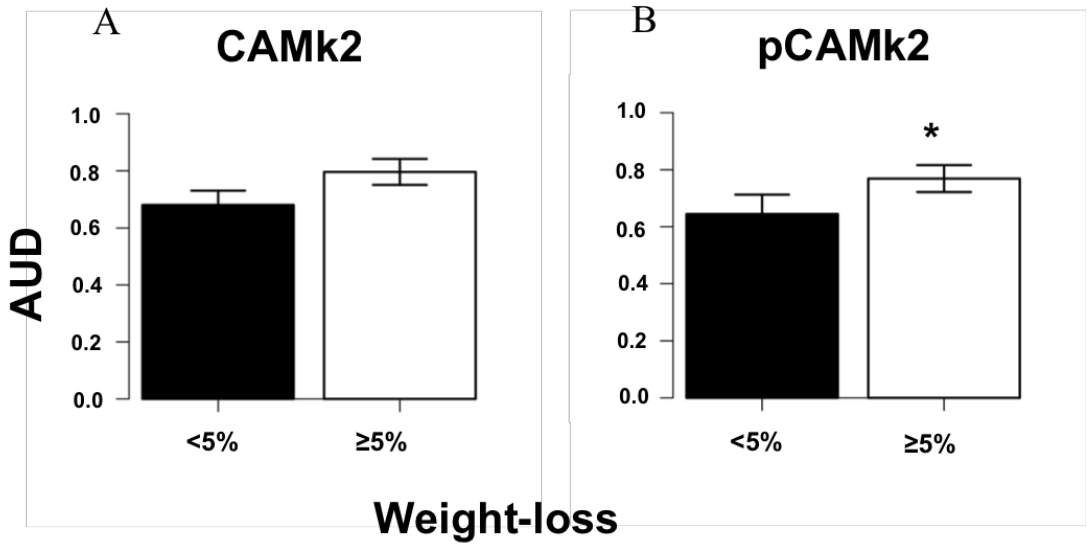
tissue from weight losing cancer patients compared with the changes noted in weight stable cancer patients and controls (p=0.039) (Figure 4.4).

Gene interaction analysis

The nature of the 82 weight-loss associated gene-signature was characterised in greater detail using Bibliometric analysis (Ingenuity database) to establish the relationship between the weight-loss correlating genes and the known biological interactome. In order to generate valid pathway or ontological enrichment scores it is essential to relate the modulated gene list with the genes detectably expressed in the tissue of interest and not with the genome as a whole (or the entire gene-chip content). This is because the ontological profile of the tissue specific expression will already be substantially enriched and bias the enrichment analysis of the regulated gene list. The analysis indicated that metabolic pathways relating to Aminophosphonate, Tryptophan, Arginine/Proline and Ubiquinone Biosynthesis were significantly over-represented in the weight-loss gene list after correction for multiple testing (Fishers Exact Test, $p<0.002$ – $p<0.001$).

Each of these categories was, however, driven by a small number of overlapping genes (PCYT2, PRMT5, EDF1, WARS, GCDH, NDUFS5, P4HB, PRODH and PYCR2). For example, WARS catalyzes the aminoacylation of tRNA(trp) with tryptophan, an essential function of the cell's protein synthesis machinery while GCDH, PRODH and NDUFS5 are mitochondrial located proteins. PRODH (Proline dehydrogenase) can enhance reactive oxygen species and degrades Proline while

FIGURE 4.4: PROTEIN LEVELS OF CAMK2 AND PCAMK2 IN CENTRE 1 PATIENTS



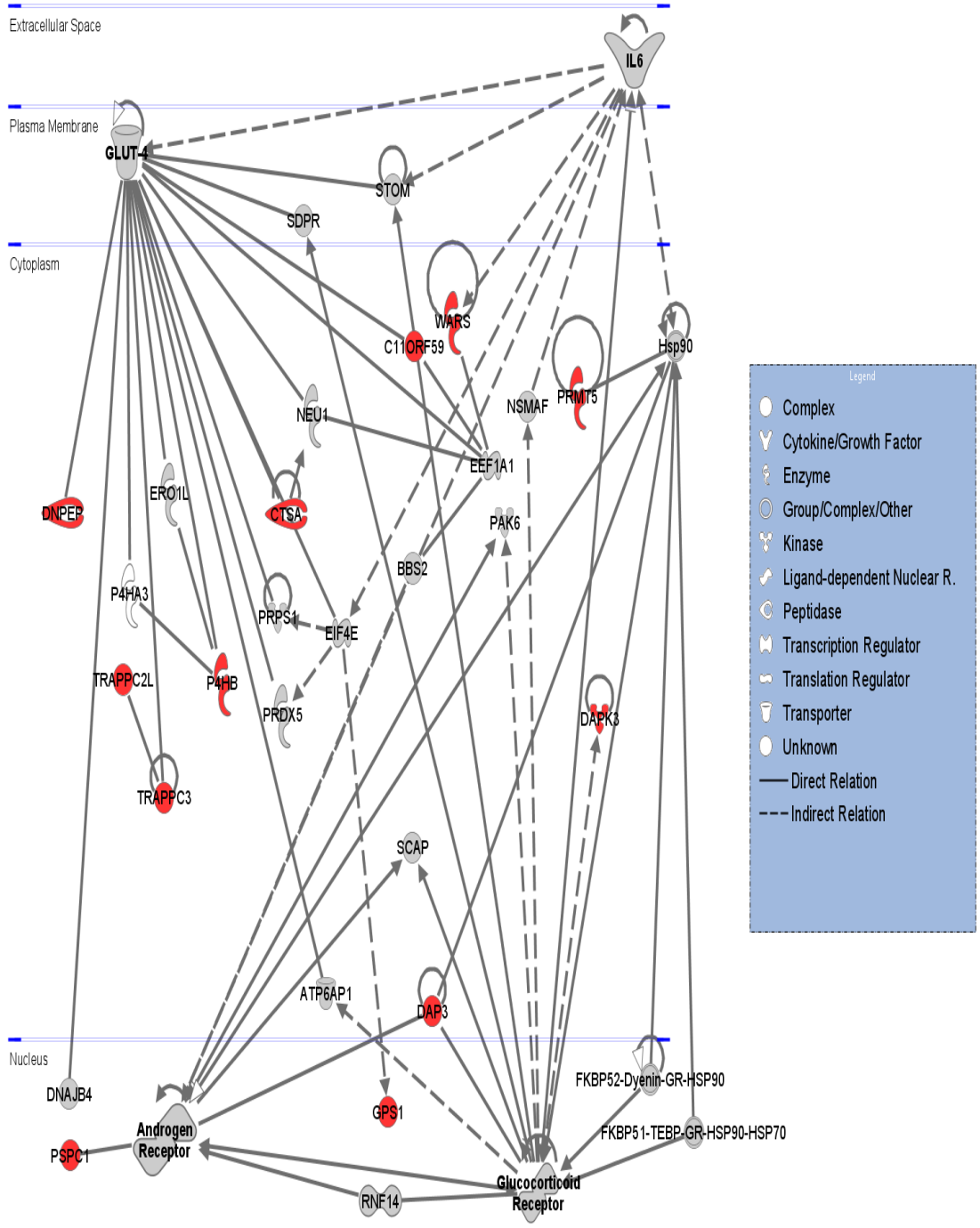
*CAMk2 protein and phospho-protein levels are increased in subjects with weight loss. Protein levels of CAMk2 (**A.**) and pCAMk2 (**B.**) were assessed in the Rectus abdominis muscle from centre 1 subjects by Western blot. Intensity levels were normalised to loading control (α-skeletal actin) and the mean AUD of CAMk2/actin or pCAMk2/actin are shown for subjects with less than (black) or more than (white) 5% weight loss. * = p-value <0.05 compared with <5% weight-loss group. Abbreviations: pCAMK2, phosphorylated CAMK2; AUD, arbitrary units of densitometry.*

P4HB (prolyl 4-hydroxylase) is involved with the hydroxylation of prolyl residues in preprocollagen and thus an essential extracellular matrix regulating gene.

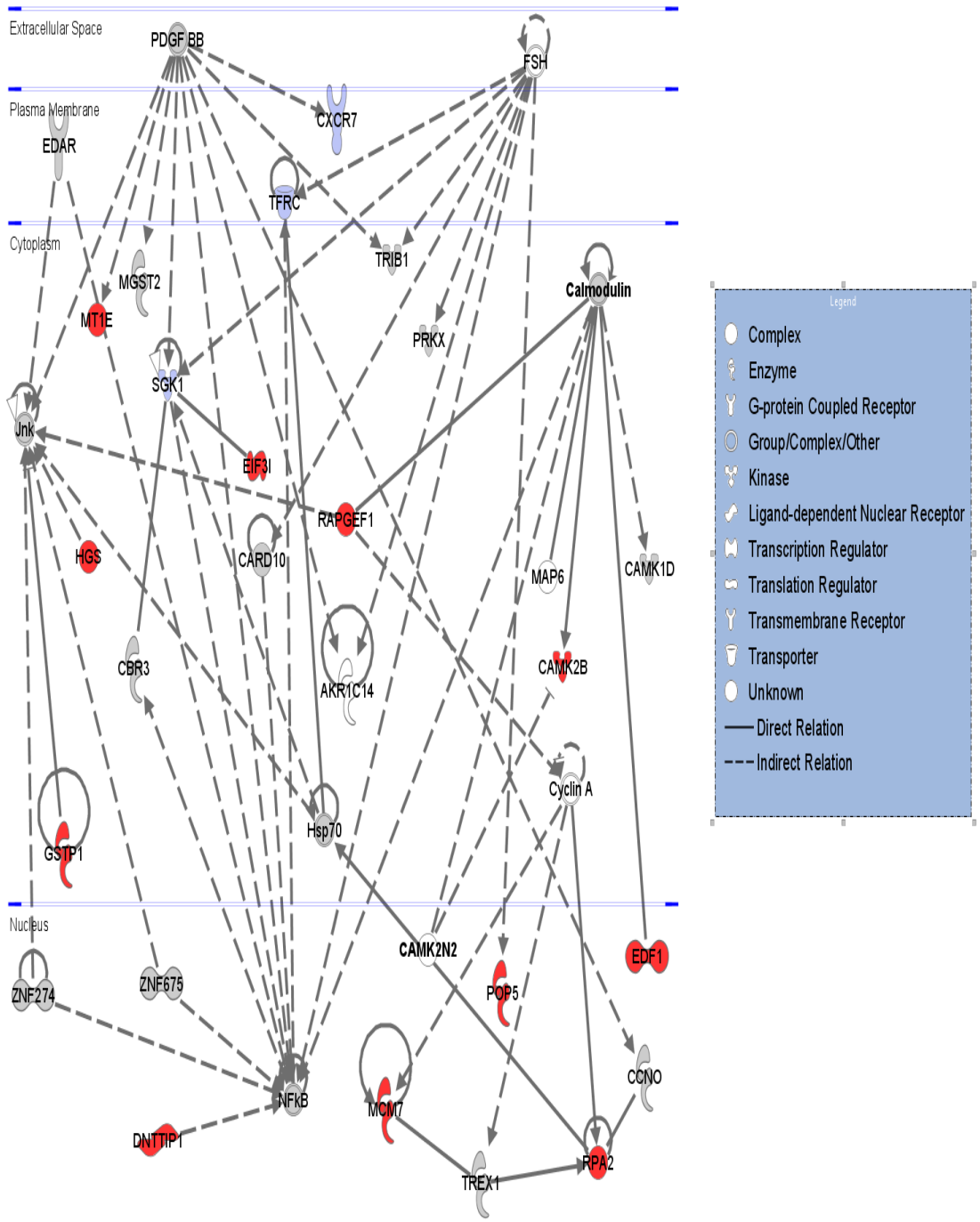
Network analysis revealed several pathways for the weight-loss associated genes including CAMk and IL-6 related gene networks (Figure 4.5). Identification of a Calcium/Calmodulin related gene network supports the wet-lab data and indicates that CAMk2β appears to be a universal marker of muscle wasting in cancer cachexia. The second pathway features Glut-4 and IL-6, both of which are implicated in skeletal muscle metabolism as well as IL-6 being a prominent pro-inflammatory mediator. This network forms numerous connections with the glucocorticoid and androgen receptors that are directly involved in regulating skeletal muscle mass. It should be noted that despite using a back-ground gene expression file in Ingenuity for genes only detected as being expressed in human skeletal muscle (~21,000 probe-sets, based on MAS present-marginal calls) the Ingenuity network analysis still included additional genes and should be used in a qualitative fashion.

FIGURE 4.5: GENE NETWORKS INVOLVING THE WEIGHT-LOSS CORRELATING GENES

A.



B.



*Using the Ingenuity database, weight-loss correlating genes were examined for gene interactions. This analysis revealed networks involving IL-6 (**A.**) and calmodulin (**B.**). Red symbols represent genes upregulated and blue symbols represent those downregulated in the weight losing group by Affymetrix analysis.*

4.6 Discussion

In this study, the expression of 82 genes correlated with weight-loss in cancer cachexia subjects; 73 of these were positively correlated and 9 were negatively correlated. Validation of the weight-loss correlating genes by qRT-PCR provided excellent technical confirmation of the microarray results. Biological validation of TIE1 and the trend in CAMk2β expression in an independent clinical cohort across distinct muscle groups has identified potential novel biomarkers of cancer cachexia in humans.

CAMk2β - a biomarker of human cachexia

The significant correlation of CAMk2β mRNA expression with weight-loss along with the small but significant change in phosphorylated protein levels in *Rectus abdominis* suggests that CAMk2β could be a useful biomarker of human cancer cachexia. There was also a positive trend of CAMk2β with weight-loss in *Vastus lateralis* and *Diaphragma* and although not reaching statistical significance, it is possible that the smaller sample size in this group gave rise to a type II error.

The serine/threonine kinase CAMk2 holoenzyme is activated by calcium (Ca^{2+})/ calmodulin leading to autophosphorylation, rendering the kinase constitutively active and maintaining CAMk2 activity even after the Ca^{2+} signal has diminished (Chin, 2004). Thus oscillatory Ca^{2+} signals can be converted into a sustained signal to activate transcription factors or other processes. CAMk2β is expressed in skeletal muscle and levels of the protein as well as phosphorylation status and activity increase after exercise training (Rose et al., 2007). The relationship between

CAMk2β expression and cachexia observed in the present study suggests that muscle wasting is not simply in response to physical inactivity. The significant positive correlation for TIE1 mRNA expression with weight-loss in both the *Rectus abdominis* and the *Vastus lateralis* muscle groups supports this claim. In animal models TIE1 is required for normal vascular network development (Sato et al., 1995). Increased TIE1 mRNA levels in human skeletal muscle in response to physiological adaptation to exercise training has been demonstrated (Timmons et al., 2005a) again underlining that the present findings are not due to illness related inactivity. Whilst the ligands and signalling pathways of TIE1 are poorly understood this receptor can interact with PI3K and leads to phosphorylation and activation of Akt, protecting cells from apoptosis (Kontos et al., 2002). In functional terms, the up-regulation of TIE1 may therefore represent a protective mechanism to oppose apoptosis.

The increased CAMk2β mRNA levels associated with weight-loss across a range of muscle tissues implies that these muscle groups have either developed dysregulation of calcium sensing or are burdened by greater loading in the face of failing muscle function. CAMk2 has also been implicated in muscle growth through phosphorylation of HDAC5 leading to MyoD/MEF2 driven differentiation of muscle cells (McKinsey et al., 2000). It is therefore plausible that CAMk2 activation is a compensatory strategy in the face of failing protein synthesis.

Alternatively, the CAMk2β response may indicate activation of calcium driven proteolytic activities such as calpains and caspases (Jones et al., 2004, Menconi et al., 2004). The UPP is considered to be the major pathway for myofibrillar protein

breakdown, but it is thought that the calpains are necessary for release of myofilaments prior to entry into the proteasome (Goll et al., 2008). A link between these pathways has been demonstrated in myotubes. Treatment with calcium ionophores led to increased proteosome activity whilst treatment with inhibitors of CAMk2, caspases and calpains all reduced this proteolytic activity (Menconi et al., 2004). It is thus possible that CAMk2β activation occurs at an early stage of cachexia in humans reflecting initiation of the calpain pathway prior to UPP driven proteolysis.

Finally, recent work has clarified two potential calcium independent activation pathways for CAMk2. Generation of reactive oxygen intermediates can increase or prolong CAMk2 activity, perhaps through inhibition of protein phosphotases which normally limit CAMk2 activation (Howe et al., 2004). Given the proposed role of inflammatory stimuli in cachexia, this could represent an important activation pathway for CAMk2. In addition, it has recently been demonstrated that Ca^{2+}-CaM-eIF2K signalling is responsible for exercise induced inhibition of muscle protein synthesis (van Hoek et al., 2009). It is conceivable that chronic inappropriate activation of so-called 'endurance specific' related signalling processes (Timmons et al., 2005b) is limiting maintenance of skeletal muscle mass. This speculation would be supported by the significant elevation in TIE1 expression. However, it is notable that (other than TIE1, CTSA and PRODH) the weight-loss gene-signature does not overlap with the ~500-strong human endurance training gene-expression signature (Timmons et al., 2005b) indicating that the genuine reason for elevated CAMk2β remains to be determined.

Proteolytic pathways in human cancer cachexia differ from pre-clinical models

Given the robust increase in expression of the E3 ligases reported previously in various animal models of cachexia (Bodine et al., 2001a, Gomes et al., 2001, Lecker et al., 2004), it is surprising that neither the microarray (this Chapter) nor qRT-PCR (as discussed in Chapter 3) results detected any significant difference in the expression of MuRF1 and MAFbx between controls and cachectic patients. Furthermore, the 82 weight-loss gene signature bore no resemblance to the Atrogen signatures (Lecker et al., 2004, Fredriksson et al., 2008). This is not due to gene-chip technology being unable to establish parallels, as it has previously been demonstrated that skeletal muscle of ICU patients resembles, in part, the process found in animal models (Fredriksson et al., 2008, Lecker et al., 2004). Results of E3 ligase expression analysis from other human models of cachexia have been contradictory. Studies including patients following bed rest, amputation for vascular disease, limb immobilisation, COPD, amyotrophic lateral sclerosis and ageing have demonstrated both increased and decreased expression of MuRF1 and MAFBx (Doucet et al., 2007, Salanova et al., 2008, Leger et al., 2006, de Palma et al., 2008, Edstrom et al., 2006). This would suggest that the ubiquitin E3 ligases do not play the same role in human cancer cachexia as that previously demonstrated in animal and cell studies.

However, cancer cachexia encompasses a spectrum progressing from early weight-loss through to severe muscle wasting with resultant incapacity, increased morbidity and mortality (Fearon, 2008). The prominence of the individual proteolytic pathways at different time points along this spectrum is yet to be determined. Investigation of the UPP in the MAC16 murine model and pancreatic cancer patients

suggests that UPP activity is reduced with low weight-loss and increases as weight-loss becomes more severe (Khal et al., 2005a, Khal et al., 2005b). Indeed, in lung cancer patients with mean weight-loss of 2.9%, there was no evidence of UPP activation (Jagoe et al., 2002). However, other human studies in patients with gastric cancer and mean weight-loss of 5.2% and 5.6% have shown increases in components of the UPP (Bossola et al., 2001, Bossola et al., 2003). Thus, the role of the UPP in human cancer cachexia could vary according to the degree of muscle wasting or may be tumour type specific. Recently a third E3 ligase (E3α-II) associated with muscle atrophy has been described (Kwak et al., 2004). This protein was identified in two animal models of cancer cachexia but has not yet been investigated in human disease and may play a role in the human muscle cachexia process.

The autophagy pathway can be activated in muscle atrophy, but its role in human cancer cachexia has not been investigated extensively. Increased cathepsin D and acid phosphatase activity has been demonstrated in patients with varying tumour types and degrees of weight-loss suggesting that increased lysosomal activity may be important in the development of cachexia (Schersten and Lundholm, 1972). More recently, lung cancer patients undergoing resection were shown to have increased mRNA levels of cathepsin B in skeletal muscle compared with controls (Jagoe et al., 2002). The current microarray analysis showed increased expression of GABARAPL1 associated with SI in cachectic patients and qRT-PCR (Chapter 3) demonstrated increased mRNA expression of both GABARAPL1 and BNIP3 in cancer patients compared with controls. GABARAPL1 is an Atg8 homologue important in the formation of the autophagosome (Tanida et al., 2004) and BNIP3

has been found to play a predominant role in induction of autophagy in rodent skeletal muscle (Mammucari et al., 2007). The role of autophagy in the cell is varied and includes programmed cell death, organelle turnover and protection against metabolic stress and DNA damage. Autophagy can be induced by starvation of amino acids, which may explain the increase in BNIP3 and GABARAPL1 in cancer patients where the acute phase response is activated (mobilising amino acid from muscle to liver for consumption) and where food intake may be reduced due to anorexia or dysphagia. Human cancer cachexia is a chronic wasting process and weight-loss is not as rapid and generally not as severe as it is in the acute muscle wasting seen in the animal models. Thus the physiological regulators may be distinct in each scenario. Whilst this study showed evidence for activation of the autophagy pathway, it was unable to replicate the finding of robust E3 ligase expression in the human data set. Neither was there increased FOXO transcription activity. Instead, there was increased expression of two 'endurance exercise' genes, CAMk2β and TIE1, across different muscle groups in human cancer cachexia. Whether this reflects activation of proteolysis via calpains prior to UPP activation, a reduction in protein synthesis or activation of alternate pathways remains to be ascertained.

The preceding two chapters have described cross-sectional biomarker studies using both a hypothesis-driven biomarker approach (Chapter 3), and a biomarker discovery approach utilising transcriptomics (Chapter 4). With cancer cachexia being a journey rather than an event, there are potential limitations that come with cross-sectional studies. As such, the next chapter will describe a further transcriptomic study, but this time, longitudinal changes will be investigated.

Chapter 5: **Investigating longitudinal changes in biomarkers of human skeletal muscle cancer cachexia**

5.1 ***Abstract***

A longitudinal transcriptomic study was carried out using microarrays to examine the global transcriptomic response in paired Vastus lateralis biopsies (baseline (pre-op: weight-loss 7%) and follow-up (8 months after surgical resection and disease-free /weight-stable for previous 2 months)) from quadriceps muscle of weight losing patients (n=12) with UGI cancer. Prior to surgery 1868 genes were regulated compared with follow-up. Ontology analysis demonstrated regulated genes belonged to both anabolic and catabolic biological processes with overwhelming down-regulation in baseline samples. Again, none of the literature-derived genes from pre-clinical cancer cachexia models demonstrated higher expression in baseline human muscle. Comparison with HC muscle revealed that despite differences in the transcriptome at baseline (941 genes regulated), the muscle of patients at follow-up was similar to control muscle (only 2 genes regulated). Physical activity (as measured by ActivPAL™ step count) did not differ between the baseline and follow-up periods (p = 0.9) indicating that transcriptome differences reflected the removal of the cancer rather than altered physical activity levels. Comparative gene-expression analysis using physical activity signatures supported this interpretation. This study showed that metabolic and protein-turnover related pathways are suppressed in weight losing UGI cancer patients whilst removal of the tumour appears to facilitate a return to a healthy state independent of changes in physical activity.

5.2 *Introduction*

Chapter 4 identified a novel gene signature relating to weight-loss. In contrast to studies in pre-clinical models, there was no evidence for an increase in proteolytic gene expression using genome-wide transcript analysis of three types of skeletal muscle in a relatively large group of weight losing UGI cancer patients. This transcriptional analysis was also supported by a lack of alteration in FOXO protein levels (Chapter 3). However protein analysis suggested that CAMk2β activation might play a role and be useful as a biomarker of cancer cachexia. In general, observations from cross-sectional studies are impacted by patient heterogeneity, including different phases of cachexia. Nonetheless, Chapters 3 and 4 seemed to suggest that human cancer cachexia is not simply 'runaway' proteolysis. Examining longitudinal changes in the skeletal muscle of cachectic patients is likely to provide further clarification.

The original intention was to undertake a longitudinal natural history study of the development of cancer cachexia in patients following resection. It was anticipated that as patients developed recurrent disease cachexia would become more prevalent and a repeat muscle biopsy could be carried out. As it happened, patients who returned for follow-up were still disease free whereas those that developed recurrence (and presumably cachexia) died so quickly as not to be picked up during follow-up.

In order to make sense of the data, a repeat muscle biopsy was carried out in those patients who were ‘cured’ and clinically disease free at follow-up assessment.

Molecular profiling of the biopsies taken before and after surgery allowed examination of the relevance of tumour burden *per se* which would potentially be informative.

Patients undergoing UGI cancer surgery lose 7-15% of body weight on average (Liedman et al., 1997). It is generally accepted that it will take 6 months for this impact of surgery to resolve (Blazeby et al., 2000, Blazeby et al., 2005a, Zieren et al., 1996, Brooks et al., 2002, Niedergethmann et al., 2006, Liedman et al., 1997). Thus, follow-up for patients in this study was at an average of ~8 months post-operatively to try and control for the potential confounding influence of the surgery. Similarly, an individual's level of exercise and physical activity will be a key determinant of muscle protein turnover. Therefore, it was felt informative to assess this in the current study. Quadriceps strength (isometric knee extensor strength (IKES)) and daily step count (ActivPAL™ monitor) were measured in UGI cancer patients at baseline and at follow-up assessment.

This chapter outlines a study investigating the global transcriptional profile of human quadriceps muscle in patients before and after potentially curative surgery for UGI cancer. Longitudinal changes in expression of the E3 ligases and autophagy genes were also examined because, although results from the cross-sectional studies of Chapters 3 and 4 have not suggested a major role in human cancer cachexia, there is some evidence that components of the UPP may be activated in a time-dependent manner (Khal et al., 2005a). Furthermore, whereas the studies in Chapters 3 and 4 used *Rectus abdominis* muscle, the longitudinal study used quadriceps (*Vastus*

lateralis) muscle which allows for repeat muscle biopsy under local anaesthesia. It is possible that different muscles have different responses to pro-cachectic stimuli and it was thus felt that a further assessment of these markers in a different muscle group would be important.

5.3 ***Hypotheses***

- There would be differences in the transcriptomic signature in skeletal muscle of cachectic UGI cancer patients before and then after potentially curative surgery (i.e. at a time when tumour-driven cachexia should have resolved).
- There would be a lack of evidence for alterations in the atrogenes in quadriceps muscle in human cancer cachexia

5.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

Muscle biopsy (*Vastus lateralis*) and blood collection: see Section 2.8

Blood measures (albumin and CRP): see Section 2.9

Muscle measures (RNA isolation, qRT-PCR, microarray target preparation/ hybridisation): see Section 2.10

Muscle function (IKES): see Section 2.3

Physical activity (step count – ActivPAL™): see Section 2.5

Statistics (general and microarray): see Section 2.12

5.5 ***Results***

Demographics

Demographic details for the UGI cancer patients before and 8 months after potentially curative surgery and for the healthy controls are shown in Table 5.1.

At baseline, age was not significantly different between UGI cancer patients and controls. BMI and weight in the cancer group did not differ significantly from the control group at any time-point. The median time between baseline and follow-up biopsies was 8 months (range 5-12 months). All UGI cancer patients were clinically cancer-free at the time of repeat biopsy. Mean weight-loss from self-reported usual weight was 7.4% at baseline and increased to 13.8% at follow-up. However, this did not occur in a linear fashion, but rather was more rapid immediately following surgery before stabilising in the two months prior to follow-up biopsy (Figure 5.1).

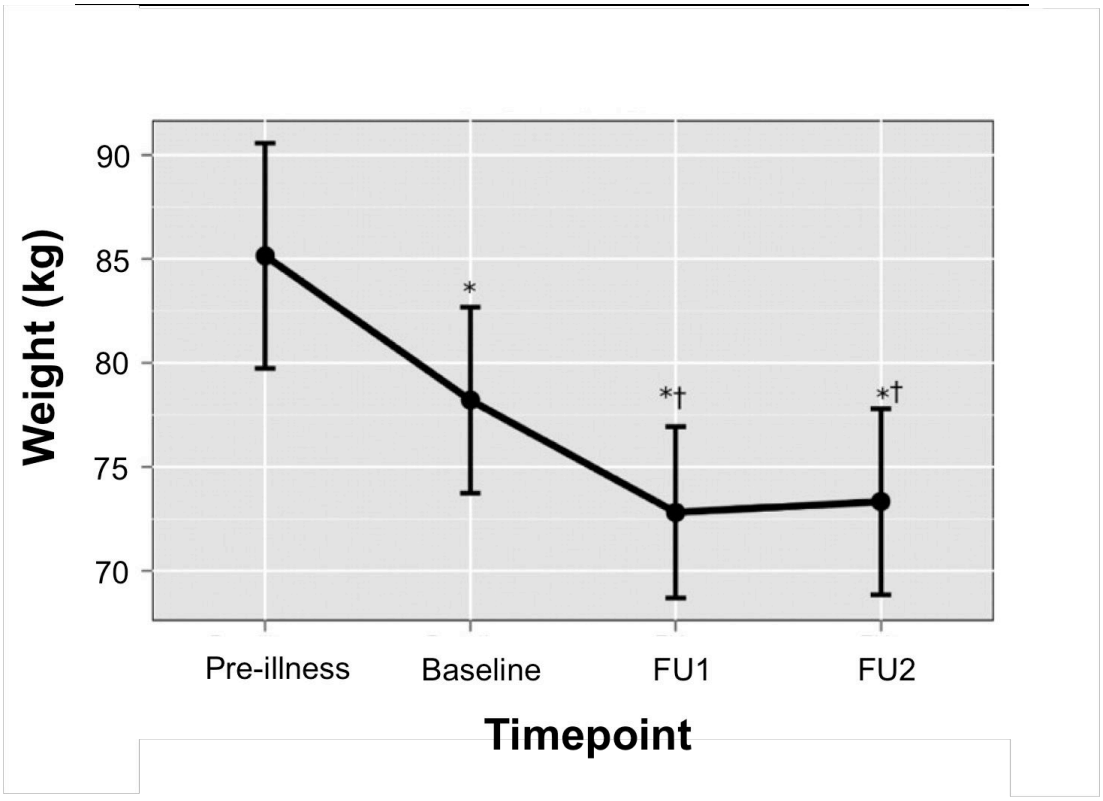
Eight of the twelve UGI cancer patients were included in the activity meter study, and age, BMI, and weight did not differ significantly from the overall UGI cancer group (data not shown). In UGI cancer patients, there was an increase in quadriceps strength between baseline and follow-up of ~20%, but this did not reach statistical significance. Overall physical activity (step count) was unchanged between the baseline and follow-up biopsy time-points (Table 5.1, Figure 5.2).

Table 5.1: Demographics, blood results, physical activity and strength for cancer patients (baseline and follow-up) and controls

	Cancer patients (paired)		Controls
	Baseline (n=12)	Follow-up 2 (n=12)	(n=6)
M/F	10/2	10/2	4/2
Age (years)	65 (3)	65 (3)	58 (6)
BMI (kg/m^2)	26.4 (1.3)	24.6 (1.3)*	28.2 (1.7)
Weight (kg)	78.2 (4.5)	72.8 (4.4)	84.9 (7.2)
Weight-loss (%)	7.3 (2.7)†	13.8 (2.7)*†	0
CRP (mg/l)	9.8 (3.6)	8.9 (4.3)	1.8 (1.0)
Albumin (g/l)	36 (1)†	42 (1)*	42 (1)
Daily step count (n=8)	5607 (1083)	5675 (600)	N/A
IKES (N/kg)	3.5 (0.3)	4.5 (0.9)	N/A

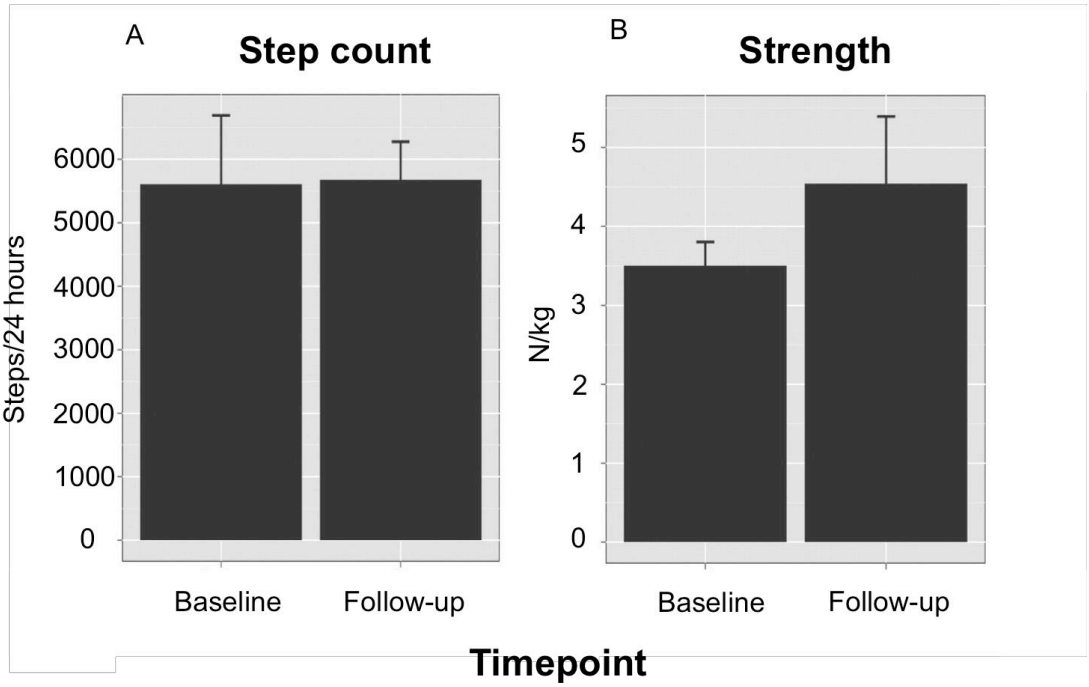
*Data (except gender split) are presented as mean (SEM). † = p<0.01 compared with controls, * = p<0.01 compared with baseline (paired t-test). Abbreviations: CRP, C-reactive protein; BMI, body mass index; N, Newtons; M, male; F, female; IKES, isometric knee extensor strength; kg, kilograms; m, metres; mg, milligrams; l, litres; g, grams; N/A, not applicable.*

FIGURE 5.1: REPRESENTATIVE WEIGHT CHANGES OF CANCER PATIENTS BEFORE AND FOLLOWING SURGERY



Weight was significantly decreased from reported usual weight at baseline and both follow-up timepoints ($p < 0.05$). At both follow-up timepoints weight was lower than baseline (†, $p < 0.05$) but did not differ between the follow-up timepoints. Muscle biopsies were taken at baseline and FU2. Abbreviations: FU, follow-up; kg, kilograms.*

FIGURE 5.2: LONGITUDINAL QUADRICEPS STRENGTH AND DAILY STEP COUNT IN CANCER PATIENTS



*(**A.**) Mean step count over fours days was not different between the baseline and follow-up timepoints. (**B.**) Quadriceps strength (IKES) did increase by ~20% at follow-up compared with baseline, but was not statistically significant. Abbreviations: N, Newtons; kg, kilograms; IKES, isometric knee extensor strength.*

Albumin and CRP

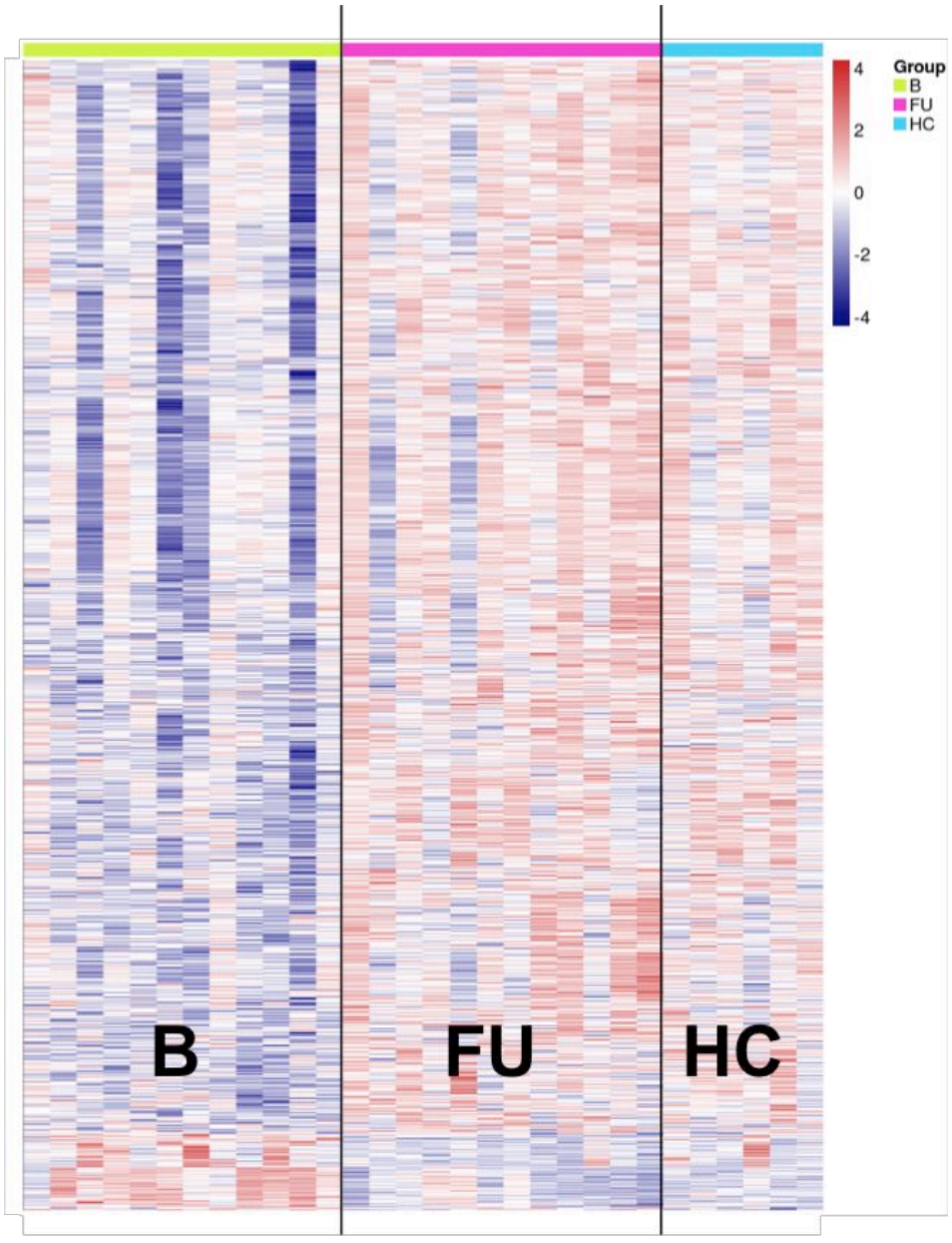
At baseline, UGI cancer patients had reduced albumin levels compared with controls (36.0 (0.5) g/l vs. 42.4 (1.3), $p < 0.001$) (Table 5.1). At the time of follow-up biopsy, albumin had returned to within the reference range (36-44 g/l) (Table 5.1). CRP did not differ between baseline and follow-up (9.75 vs. 6.0 mg/l, $p = 0.5$) and was not significantly different compared with controls at either time point (Table 5.1).

Paired muscle biopsy transcriptome

Using paired samples from the UGI cancer patients (n=12) obtained immediately pre-operatively (baseline: cancer present; weight losing) and at a median of 8 months post-operatively (follow-up: cancer resected; weight stable in preceding at least two months), global gene expression was examined using microarrays. Paired samples analysis within the SAM method (Tusher et al., 2001) identified 1868 regulated genes with a FDR of 6% and a fold change of at least 30% - 1747 downregulated and 121 upregulated in baseline samples compared with follow-up samples (Figure 5.3). Expression of selected regulated genes by qRT-PCR was validated with good agreement between the microarray and qRT-PCR data (Table 5.2).

In order to examine the biological context of the regulated genes the hypergeometric t-test was used to identify enriched Gene Ontology biological processes (GOBP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Using a background list of genes detectably expressed in muscle tissue (Gallagher et al., 2010), cellular metabolic process was the most enriched biological process ($p < 0.001$).

FIGURE 5.3: CLUSTER ANALYSIS ILLUSTRATING DIFFERENTIALLY EXPRESSED GENES FOR CANCER PATIENTS (BASELINE AND FOLLOW-UP) AND CONTROLS



Expression data from the differentially expressed genes between the baseline and follow-up timepoints were used to drive cluster analysis and produce a heatmap. Gene expression is also shown for the healthy control group. Each coloured rectangle represents a different gene in an individual patient. Red represents higher expression and blue lower expression. Abbreviations: B, baseline cancer patients; FU, follow-up cancer patients; HC, healthy controls.

Table 5.2: qRT-PCR validation of selected genes from the array data

	Baseline vs. Follow-up		Healthy vs. Baseline	
	Array (FDR)	qRT-PCR (p-value)	Array (FDR)	qRT-PCR (p-value)
COMP	3.9 (4.1)	4.3 (<0.01)	0.7 (53)	0.4 (0.02)
ADIPOQ	3.3 (4.1)	3.2 (<0.01)	0.4 (48)	0.5 (0.06)
MMP3	3.2 (6.1)	2.7 (0.04)	0.7 (53)	0.6 (0.20)
PCK1	3.0 (6.1)	2.5 (0.01)	0.6 (50)	0.5 (0.14)
ANGPTL7	2.8 (6.1)	2.5 (<0.01)	0.6 (51)	0.6 (0.46)
HSP90AB1	0.5 (6.1)	0.5 (0.04)	2.2 (10)	2.3 (0.10)
SLC25A37	0.5 (6.1)	0.5 (<0.01)	2.6 (5.1)	2.6 (< 0.01)
PROX1	0.5 (2.1)	0.5 (<0.01)	2.8 (0.0)	2.6 (< 0.01)
RCAN1	0.4 (0.0)	0.5 (<0.01)	2.0 (5.5)	2.4 (< 0.01)
HINT3	0.3 (0.0)	0.2 (<0.01)	3.1 (0.0)	3.1 (< 0.01)

Array data are presented as fold change (FDR) and qRT-PCR data as fold change (Bonferroni corrected p-value for difference in means). n=12 paired samples for follow-up vs. baseline analysis; n=5 healthy control (for one sample there was insufficient RNA for qRT-PCR) and 12 baseline samples for healthy vs. baseline analysis. qRT-PCR data were analysed by paired t-tests for follow-up vs. baseline analysis and by unpaired t-tests for healthy control vs. baseline analysis. Abbreviations: FDR, false discovery rate; qRT-PCR, quantitative real-time polymerase chain reaction.

The enriched GOBP categories included several broad metabolic processes (e.g. metabolic process, primary metabolic process), muscle growth categories (e.g. striated muscle hypertrophy, regulation of muscle hypertrophy), catabolism categories (e.g. endoplasmic reticulum-associated protein catabolic process, ubiquitin-dependent protein catabolic process) and anabolism categories (e.g. response to insulin stimulus, response to protein stimulus). In each of these categories the genes involved were overwhelmingly downregulated in the baseline samples. Selected categories are presented in Table 5.3.

Enriched KEGG pathways included the Calcium signalling pathway, the Peroxisome pathway and Epidermal growth factor receptor signalling (ErbB signalling pathway). IPA was used to examine a set of 350 robustly regulated genes that did not overlap with endurance or resistance training datasets (Melov et al., 2007, Keller et al., 2011). Network analysis, relying on the IPA database revealed the proteasome, NFκB and caspases as potential network hubs connected to many down-regulated transcripts (Figure 5.2).

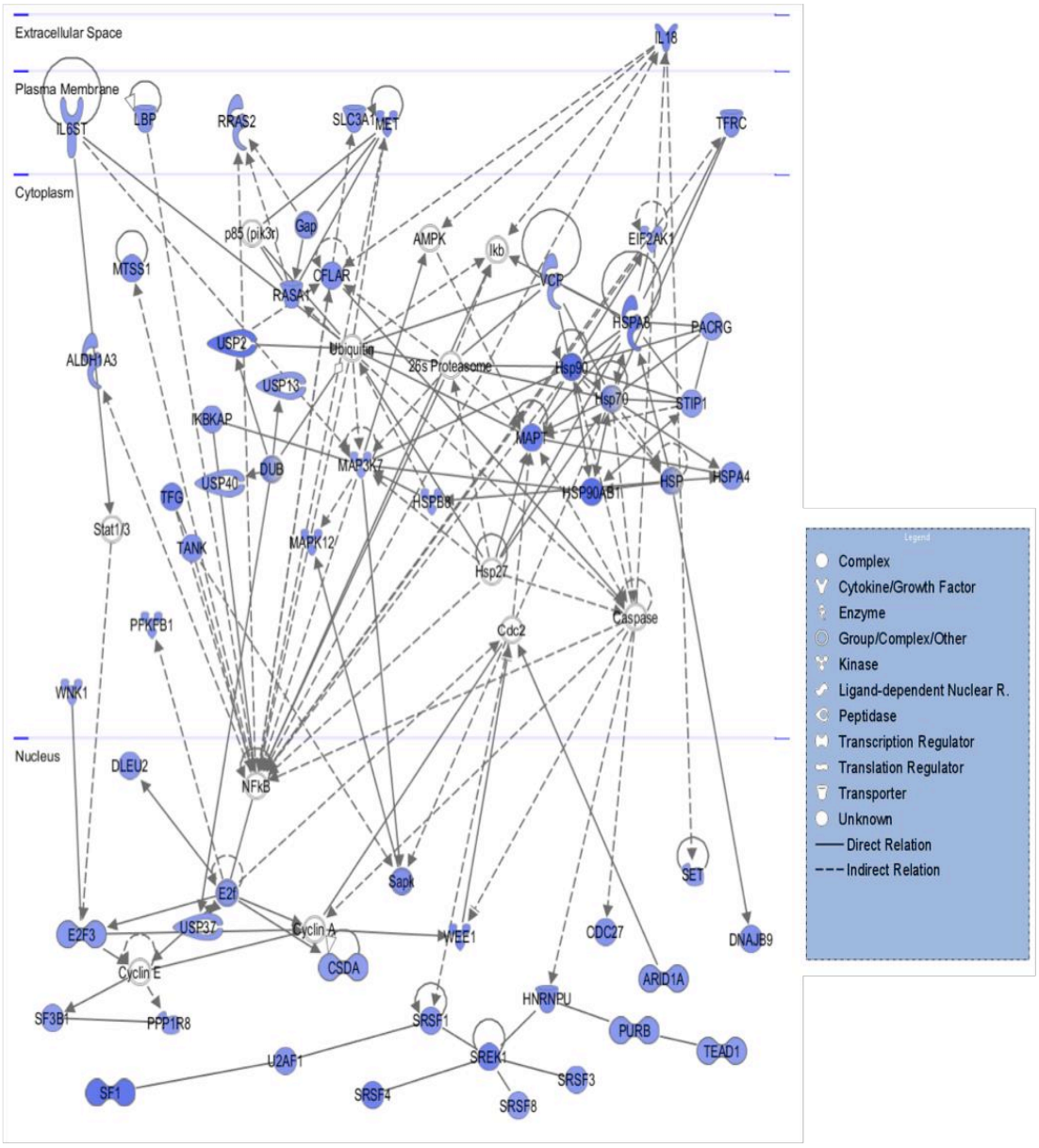
Downregulation of these network genes demonstrated that activation of the hub-genes was lower pre-operatively supporting the lack of activation of proteolytic genes in the differential expression analysis. Using gene set enrichment analysis the gene expression data for genes under the control of specific transcription factors was examined. This analysis yielded no significant results suggesting that no single canonical signalling pathway is driving the muscle cachexia profile. Pre-clinical

Table 5.3: GOBP categories regulated between baseline and follow-up timepoints in cancer patients

GOBP identifier	p-value	Count		Annotated genes on array	Term
		Expected	Actual		
GO:0044237	0.00	784	859	5042	cellular metabolic process
GO:0006350	0.01	288	320	1854	transcription
GO:0045449	0.00	277	315	1784	regulation of transcription
GO:0009056	0.03	133	153	858	catabolic process
GO:0030163	0.01	45	59	288	protein catabolic process
GO:0006511	0.01	34	48	216	ubiquitin-dependent protein catabolic process
GO:0032868	0.02	15	24	99	response to insulin stimulus
GO:0051789	0.03	13	20	85	response to protein stimulus
GO:0061061	0.04	37	47	236	muscle structure development
GO:0007517	0.01	31	43	197	muscle organ development
GO:0060537	0.04	20	28	131	muscle tissue development
GO:0014706	0.02	19	28	124	striated muscle tissue development

Abbreviations: GOBP, Gene Ontology biological processes.

FIGURE 5.4: GENE NETWORK FOR REGULATED GENES BETWEEN BASELINE AND FOLLOW-UP TIMEPOINTS IN CANCER PATIENTS



IPA analysis revealed a network involving the proteasome, NFκB and caspases as potential network hubs with overwhelming downregulation of the network genes. Blue symbols represent downregulated genes. Abbreviations: IPA, Ingenuity Pathway Analysis; NFκB, nuclear factor kappa B.

models (Bodine et al., 2001a, Gomes et al., 2001, Lecker et al., 2004) have identified a set of genes, commonly termed atrogenes, which are involved in cancer driven muscle wasting. In particular the levels of the E3 ligases MAFBx/atrogin-1 and MuRF1 are elevated in the muscle of cachectic animals. These genes were not differentially expressed in the microarray data nor when further examined by qRT-PCR (MAFBx/atrogin-1 p=1.00; MuRF1 p=0.68, paired t-test) (Table 5.4). Examination of the autophagy genes BNIP3 and GABARAPL1 (Mammucari et al., 2008, Zhao et al., 2008) also revealed no changes in expression of these genes between baseline, follow-up or healthy control groups (Table 5.4).

Physical activity

To control for potential changes in physical activity in the baseline versus follow-up periods, the average step count per 24 hours was measured (n=8/12 UGI cancer patients). Mean (SEM) step count/24 hours at baseline were 5607 (1210) and 5675 (917) at follow-up. This difference was not significant (p = 0.90, paired t-test) (Table 5.1, Figure 5.1).

As a complementary approach to direct measurement of physical activity, a comparison of the list of differentially expressed genes in cancer cachexia was compared with those identified recently in the response to two types of exercise training (Melov et al., 2007, Keller et al., 2011). Of the 848 endurance training responsive genes only 60 overlapped with those regulated between the baseline and follow-up time-points and even fewer overlapped with strength training.

Table 5.4: qRT-PCR data for selected atrophy-associated genes

	Baseline vs follow-up cancer patients	Baseline cancer patients vs control	Follow-up cancer patients vs control
MuRF1	0.94 (1.00)	1.22 (0.68)	1.30 (0.22)
MAFBx/atrogin-1	1.0 (1.00)	0.96 (1.00)	0.96 (1.00)
BNIP3	0.87 (0.18)	0.72 (0.22)	0.83 (0.50)
GABARAPL1	0.79 (0.26)	0.66 (0.14)	0.84 (0.70)

Data are presented as fold change (Bonferroni corrected p-value for difference between means). Baseline versus follow-up data was analysed using paired t-tests. Abbreviations: qRT-PCR, quantitative real time polymerase chain reaction.

In order to compare the response in cancer cachexia with that seen in dietary restriction, the overlap with the 2839 differentially expressed genes identified in response to simple dieting (Larrouy et al., 2008) was also examined. Only 12 genes overlapped between the current study and those regulated in skeletal muscle in response to simple dieting.

Thus multiple array analysis strategies demonstrated that the major phenotype of skeletal muscle 'responding' to removal of a tumour does not appear to relate to changes in physical activity or nutritional status.

Comparison with healthy control muscle

Comparison of the muscle from the UGI cancer patients at both the baseline and follow-up timepoints with muscle from weight stable healthy control subjects revealed 941 genes with lower expression (FDR <10%) in baseline UGI cancer muscle compared with healthy control muscle. At baseline no genes were expressed at higher levels in UGI cancer patients compared with control muscle with FDR <10%. Of the 941 downregulated genes, 558 were also regulated between the baseline and follow-up samples and all except one (TFDP2 - transcription factor Dp-2) were higher at follow-up. Highly enriched GOBP categories included lipid oxidation, catabolic process, protein polyubiquitination, muscle structure development and striated muscle hypertrophy. Enriched KEGG pathways included fatty acid metabolism, metabolic pathways, peroxisome, oxidative phosphorylation, ubiquitin mediated proteolysis and regulation of autophagy.

Comparison of gene expression between control muscle and the follow-up samples revealed only two genes differentially expressed within the FDR cut-off (NR1D2 & HDAC9). Thus the follow-up samples had a transcriptomic profile indistinguishable from healthy muscle albeit that the total sample size was limited for this unpaired analysis. This was also visually represented by cluster analysis (Figure 5.3).

5.6 Discussion

This study examined the transcriptomic response of skeletal muscle before and after potentially curative surgery for UGI cancer. Resection of UGI cancer is routinely associated with a weight-loss of 7-15% of body weight (Liedman et al., 1997) and patients will generally not regain nor return to their pre-operative weight. Weight-loss during the post-surgical phase is considered to be largely due to loss of fat mass with only limited changes in muscle mass (Liedman et al., 1997) suggesting that greater emphasis should be placed on the restoration of muscle phenotype and function rather than body weight. The present study investigated patients undergoing tumour resection with an average weight-loss of 7% body weight (i.e. fulfilling the diagnostic criteria for cancer cachexia (Fearon et al., 2011)). Patients underwent a pre-operative baseline quadriceps biopsy with a repeat quadriceps biopsy performed approximately 8 months later when all patients were clinically disease-free and had been weight stable for at least 2 months. It is thus reasonable to consider that the patients in the present study had recovered from the net catabolic effects of both their cancer cachexia and surgery. Sequential paired biopsies allowed analysis of changes in skeletal muscle phenotype during recovery while comparison with a group of weight-stable healthy controls allowed determination of the completeness of recovery. In the present study, patients with UGI cancer demonstrated normalisation of muscle phenotype post-surgery. Strikingly neither the cancer cachexia signature nor the recovery of healthy muscle phenotype related to levels of physical activity.

The present study demonstrated that in human skeletal muscle, of the 1868 regulated genes associated with cancer and weight-loss, the vast majority (94%) were

downregulated. Category Analysis of the differentially expressed genes demonstrated that both anabolic (e.g. muscle organ development, response to insulin stimulus) and catabolic (e.g. ubiquitin-dependent protein catabolic process, regulation of proteasomal protein catabolic process) processes were suppressed. Furthermore there was almost a complete lack of overlap with transcriptomic signatures from endurance training, strength training or simple dieting (Melov et al., 2007, Keller et al., 2011, Larrouy et al., 2008, Timmons et al., 2005a). This is perhaps not surprising as each of these situations may be considered within the range of normal physiology rather than pathologic states. The current study identified the inflammatory transcription factor NF-κB as a hub gene using IPA, however, this gene is linked to many aspects of muscle remodeling including responses to endurance training. Other approaches to identify transcription factors (*a priori* gene set enrichment analysis and *post hoc* analysis with the web-based oPOSSUM software) did not yield any significant results, suggesting that no single transcription factor network is involved in human cancer cachexia. Limited numbers, variable human data, and transcription factor redundancy are factors that might constrain the discovery of such underlying control genes.

Comparison of the UGI cancer patients with weight stable non-cancer controls showed that following removal of cancer and stabilisation of weight-loss, the transcriptome essentially returns to normal. Taken together, these observations suggest that net muscle turnover is suppressed in the early stages of cancer cachexia, but with successful cancer treatment, these changes are reversible. These observations may have implications for the timing of interventions such as physical

therapy, as it may be more productive to intervene during this stable period when the molecular phenotype is apparently healthy. Alternatively, it is possible that the recovery of the molecular phenotype could be accelerated with such interventions at an earlier stage. Future studies may be able to address this point.

In a recent study on the response of adipose tissue to human cancer cachexia, 364 genes were downregulated and 61 genes upregulated in abdominal subcutaneous white adipose tissue (Dahlman et al., 2010). Similar to the present study pathway analysis indicated that downregulated genes were involved in cytoskeleton and extracellular matrix processes. Changes in gene expression were reciprocal to those observed in obesity suggesting that the effects of reduced food intake may dominate regulation of fat mass in cachexia. In the present study, the gene changes in muscle were dissimilar to those seen in simple dieting (Larrouy et al., 2008), thereby confirming the essential phenotype of cachexia in that the response of muscle to cancer is distinct from simple starvation (Nixon and Lawson, 1983, Fearon et al., 2011) and cannot be explained by cancer-induced anorexia alone. This observation may also explain the sub-optimal skeletal muscle response to nutritional supplementation observed in cancer patients.

The transcriptomic signal that was detected at baseline in UGI cancer patients suggests depression of muscle turnover in patients with cancer-associated weight-loss supporting earlier ideas that both global protein synthesis and breakdown were suppressed. Whilst muscle turnover could be a function of changes in physical activity there did not appear to be any differences in 24-hour step count between

baseline and follow-up assessments. Furthermore, the genes differentially expressed after removal of the tumour showed little overlap with genes expressed after exercise training suggesting that increased turnover was not simply a re-training response. The median time interval between operation and follow-up biopsy was 8 months and it is generally considered that muscle function would be recovered by this time (Mathur et al., 2008).

The loss of skeletal muscle protein in cancer cachexia has been attributed to both alterations in the transcription of specific genes leading to changes in muscle turnover and/or a global reduction in cellular RNA reducing overall translational capacity. A global reduction in RNA abundance might be due to reduced transcriptional capacity as a result of loss of myonuclei secondary to enhanced apoptosis or reduced satellite cell recruitment. Evidence of apoptosis has been reported in cachectic patients with cancer similar to those involved in the present study (Busquets et al., 2007). However, Lundholm and colleagues reported that human skeletal muscle RNA content is unaffected by the presence of a tumor, whereas in animal models, there was a decrease in RNA content (Lundholm et al., 1978). While animal models carrying a variety of tumors have shown reduced RNA content in muscle (Emery et al., 1984b, Baracos et al., 1995, Bhogal et al., 2006) in two of these models, there was also evidence for concurrent increases in mRNA expression of proteasomal subunits (Khal et al., 2005b, Baracos et al., 1995). Notably, this Chapter has demonstrated a reduction in mRNA expression for proteasomal pathway components in human UGI cancer and similarly, Chapter 4 did not show any evidence for changes in mRNA for these components.

Direct evidence for the involvement of specific proteolytic and synthetic pathways in human cancer cachexia is limited. However the balance of evidence, based on stable isotope and global transcript analysis appears to favour the conclusion that suppression of both processes occurs, with the reduction in synthetic pathways exceeding suppression of the proteolytic pathways. In line with this, Lundholm and co-workers used net release of 3-methylhistidine across the leg to examine protein degradation and found rates lower in cancer patients compared with healthy controls or acutely ill patients. Reduced protein breakdown was also accompanied by anabolic blunting in response to feeding suggesting a net catabolic status rather than increased degradation (Lundholm et al., 1982). Dworzak and co-workers subsequently reported depressed skeletal muscle protein synthesis in the face of maintenance of protein breakdown, again suggesting relative catabolic advantage (Dworzak et al., 1998). Emery and co-workers found evidence of reduced protein synthesis (protein breakdown was not measured) using labelled leucine in cancer patients and suggested that part of this synthetic depression was related to reduced translation (Emery et al., 1984a). Mechanistically this may reflect increased levels of phosphorylated, inactivated eIF2 in weight losing cancer patients compared with healthy controls (Eley et al., 2008). Together these data suggest that whilst protein degradation may play a selective role in human cancer-associated muscle wasting (where specific proteins are targeted), the overall role of this pathway is much less than that seen in pre-clinical model systems, and depressed protein synthesis probably contributes more to a net increase in muscle loss. This paradigm is consistent with the findings of the present study that suggest an overall decrease in muscle turnover in cancer cachexia in humans.

What remains to be determined is the precise nature of the cross-talk between the tumour and the process of skeletal muscle cancer cachexia. Certain transcript changes in the present study might be explored as biomarkers of cancer cachexia and provide insight into the nature of the process. Increased serum cartilage oligomeric matrix protein (COMP) gene expression in cachectic muscle was seen in the current study and a similar pattern of expression has been seen in muscle damage in marathon runners (Kim et al., 2009). Similarly expression of matrix metallopeptidase 3 (MMP3), also increased in cachectic muscle, is increased in models of muscle injury (Goetsch et al., 2003). These findings are consistent with the suggestion that removal of cancer allows skeletal muscle to recover from net catabolism. There is also recently published data detailing a role for ADIPOQ, upregulated at baseline, as a network hub in cachexia (Tan et al., 2011) suggesting that this might have a role in cancer-associated muscle atrophy or be a sensitive integrator of the net underlying processes e.g. metabolic status of the muscle. Increased expression of the phosphoenolpyruvate carboxykinase 1 (PCK1) gene has been found in lean rodents (Novak et al., 2009) where it has been linked to increased endurance capacity. However the finding of increased PCK1 expression in cachectic muscle in the current study may suggest that this is representative of a possible compensatory response in the face of catabolic advantage.

Of the genes downregulated at baseline, regulator of calcineurin 1 (RCAN1) inhibits calcineurin signalling and is upregulated in muscle adapting to eccentric exercise (Mahoney et al., 2008) and calcineurin activation may be a key feature of muscle adaptation to endurance exercise in humans (Keller et al., 2011). Again these

changes suggest that the muscle is attempting to compensate for the influence of the tumour, supporting the conclusion that muscle remodelling/turnover is specifically suppressed by the presence of cancer. In rodents inactivation of the transcription factor prospero homeobox 1 (PROX1) leads to disorganisation of cardiac muscle (Risebro et al., 2009). If this function were recapitulated in skeletal muscle then reduced PROX1 expression would indicate a failure of proper muscle remodelling.

The previous analysis of gene expression in muscle of cancer patients outlined in Chapter 4, demonstrated that expression of the exercise-activated genes CAMk2β and TIE1 was increased in patients with increasing weight-loss. However, although at the follow-up timepoint UGI cancer patients were weight stable from the perspective of the post-operative period, their overall weight was still lower than at the point of diagnosis. Therefore, it is possible that CAMk2β and TIE1 upregulation are indicators of muscle mass regulation; an idea consistent with the relatively clear ability of these genes to identify even modest cancer cachexia muscle changes. The association of CAMk2β and TIE1 with weight-loss in the longitudinal study suggests they are 'longer term' markers of muscle status and thus potential biomarkers for weight-loss in cancer cachexia. Nevertheless, the optimal biomarker would predict the potential for induction of cancer cachexia in the individual and to establish and validate such a biomarker would require much larger studies.

Current therapeutic approaches to the management of muscle wasting in cancer cachexia include such general measures as nutritional support and exercise interventions (Fearon, 2008). Difficulties with low compliance and negligible benefit

have stimulated much interest in measures to overcome hypo-anabolism or reduced increased catabolism. The predominance of an activated UPP driving protein breakdown in some acute animal models of muscle atrophy has led many to suggest the UPP should be targeted in patients with cancer cachexia. Whilst the UPP might be relevant at some point during the development of cancer cachexia (e.g. during a the acute phase of a period of prolonged bed-rest), the findings of the present Chapter suggest that more emphasis should be given to the stimulation of anabolism and in particular blocking of the specific effect that tumour burden has on muscle signalling. The finding that changes in the transcriptome are reversible with successful removal of the cancer suggests that optimal benefit might be observed if such cachexia therapy was provided during or immediately after oncological therapy, rather than being left to a stage when both the cancer and cachexia are refractory to intervention (Fearon et al., 2011).

Summary of Part I

Part I has focused on classification of cancer cachexia by investigating potential molecular biomarkers related to early cachexia. The first study in Chapter 3 aimed to assess selected candidate biomarkers known to be relevant in muscle wasting in pre-clinical models. Overall, there was an overwhelming lack of parallel between the pre-clinical models and human cancer cachexia. In particular, the ubiquitin E3 ligases were not related to human cancer cachexia. However, β-dystroglycan was shown to be a potential biomarker relating to weight-loss, GABARAPL1 and Akt as biomarkers of cancer/early cachexia and MyHC and dystrophin as biomarkers relating to survival. Building on these findings, the next study in Chapter 4 used global transcriptomic profiling to search for biomarkers relevant in human cancer cachexia. A novel 82 gene signature was identified which could classify patients as cachectic or not cachectic based on a weight-loss of 5%. This signature did not relate to any of the key pathways relevant in animal models. Selected genes from this list were validated in *Rectus abdominis*, *Diaphragma* and quadriceps (*Vastus lateralis*) muscle using qRT-PCR. In particular, CAMk2β and TIE-1 (endurance genes) seemed to closely relate to cachexia and CAMk2β protein levels were increased. The final study in Part 1 described in Chapter 5 used a novel longitudinal approach to investigate changes in the transcriptomic signature in patients recovering from ongoing weight-loss after curative resectional surgery. This suggested that there was relative hypo-anabolism in human cancer cachexia with blunting of both anabolic and catabolic pathways. The atrogenes did not change with time, suggesting a limited role in human cancer cachexia. Furthermore, there was no overlap between exercise/starvation signatures and the cachectic signature suggesting

that it is a *bona fide* signature of cachexia. Thus, rather than confirm the conventional view from pre-clinical models that there is strong upregulation of protein degradation in early cachexia, Part I of the current thesis suggests a depression of synthetic pathways and that changes in structural components of the muscle (β-dystroglycan) or in compensatory mechanisms (CAMk2β) may be better potential biomarkers of early cachexia in muscle. Clearly these findings need to be confirmed in different patient groups before being proposed for validation programmes.

Having described both potentially novel intramuscular biomarkers relating to cachexia, along with stark contrasts in the molecular events in skeletal muscle in humans compared with the pre-clinical literature, Part II of the thesis will focus on a series of physiological assessment studies to phenotype muscle mass and function in cancer cachexia.

Part II: **Cancer cachexia: physiological assessment of skeletal muscle**

> "Although nature commences with reason and ends in experience it is necessary for us to do the opposite, that is to commence with experience and from this to proceed to investigate the reason."
>
> Leonardo da Vinci

> "Women may fall when there's no strength in men."
>
> Act II, Scene III, Romeo and Juliet, William Shakespeare

Chapter 6: **Investigating the potential of a novel MR imaging analysis method to assess muscle and fat content in cancer cachexia patients.**

6.1 ***Abstract***

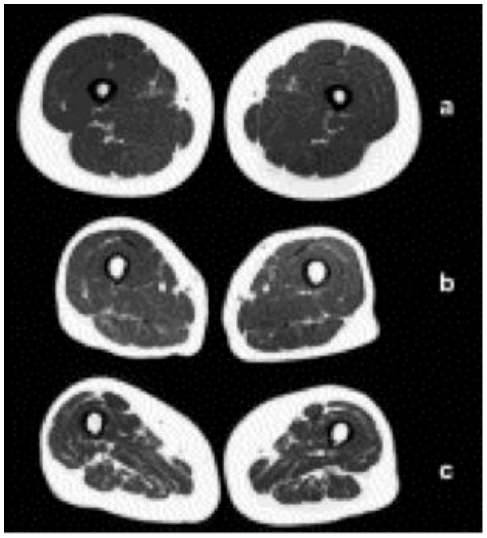
Previous studies have suggested that cachexia and sarcopenia of ageing are characterized by infiltration of non-contractile tissue within muscle (which influences area and volume measurements). This Chapter assessed whether a MR image analysis technique could provide useful insights into skeletal muscle composition and wasting in cachectic patients. A novel statistical clustering (k-means statistical clustering) technique was applied to MR images of the quadriceps of young and older healthy women and women with UGI cancer in order to separate the contractile and non-contractile tissue compartments. MR scans of the thigh were obtained for 34 women (n = 16 young, (median) age 26y; n = 9 older, age 80y; n = 9 UGI cancer patients, age 65y). Segmented regions of consecutive axial images were used to calculate cross-sectional area and (gross) volume. Women with cancer and older women had 48% and 37% less quadriceps muscle respectively than young women ($p < 0.001$). Application of k-means statistical clustering subtracted a significant 9%, 14% and 20% non-contractile tissue from the quadriceps of young, older and cancer patient groups respectively ($p < 0.001$). There was a significant effect of group (i.e., cancer vs. healthy) when controlling for age as a covariate ($p = 0.003$). The results showed that k-means statistical clustering provides an objective separation of contractile and non-contractile tissue components and is therefore a useful tool in the assessment of muscle composition in patients with cancer cachexia. Women with UGI cancer had significant fatty infiltration throughout whole muscle groups, which was maintained when controlling for age.

6.2 *Introduction*

In Part I different approaches were applied to biomarker discovery in skeletal muscle for early cachexia. The findings ran somewhat contrary to pre-clinical models and thus need further careful exploration before being considered in the context of a biomarker validation programme. Such issues emphasise the need for a dual approach to the classification of cancer cachexia: if molecular markers prove difficult to discover/validate, then more specific and robust physiological indices of skeletal muscle mass and function may be the more important route to improve clinical intervention trial design/cachexia assessment.

As discussed in the Section 1.2.4.1, the measurement of muscle mass in humans can be challenging. The increasing availability of cross-sectional imaging (MR or CT) has provided a gold standard and these techniques are able to distinguish between different tissue types, e.g. muscle and subcutaneous fat. However, these imaging modalities are not without limitations. One potential confounding factor in studies specifically investigating muscle wasting is the infiltration of muscle by fat (both inter- and intra-muscular) and/or non-lean tissue (Frantzell and Ingelmark, 1951, Forsberg et al., 1991, Overend et al., 1992, Tsubahara et al., 1995, Jubrias et al., 1997, Kent-Braun et al., 2000, Macaluso et al., 2002, Song et al., 2004) (Figure 6.1). Attempts to address this problem have been made with varying success by utilisation of post-hoc techniques such as manual design based stereology (Jubrias et al., 1997) or image analysis (Frantzell and Ingelmark, 1951, Overend et al., 1992, Tsubahara et al., 1995, Kent-Braun et al., 2000, Macaluso et al., 2002, Song et al., 2004) to

FIGURE 6.1: MR IMAGES ILLUSTRATING FATTY INFILTRATION WITHIN THE QUADRICEPS MUSCLE GROUP.



MR cross-sectional images at mid-thigh level in healthy women aged 23 years (a) and 80 years (b) and a woman with UGI cancer aged 75 years (c). Fat/non-contractile tissue can be visualised as white whereas muscle is darker grey. Abbreviations: MR, magnetic resonance; UGI, upper gastrointestinal.

separately quantify the contractile/non-contractile compartments within the 'muscle' region of interest. Ageing is associated with loss of muscle mass (sarcopenia) and MR image-derived measurements have shown that older healthy human skeletal muscle contains a two- to three-fold greater non-contractile tissue area/volume compared with younger muscle (15% vs. 6%) (Kent-Braun et al., 2000, Macaluso et al., 2002). Loss of muscle may result in poorer function with subsequent decline in physical independence and QoL (Young, 1997) and decreased survival rate following critical illness (Lee et al., 2007). In cancer cachexia, as previously discussed, skeletal muscle is a primary target. Muscle size is an important determinant of functional status and as such, accurate quantification is necessary.

However, there are few reports of the measurement of muscle size in human cancer cachexia and no focus on attempting to differentiate lean from fatty tissue. The use of CT and image analysis (a modification of a technique first reported in healthy adults) (Shen et al., 2004) to estimate whole body skeletal muscle mass and/or adiposity from CSA measurements at the level of L3 has been discussed earlier in Section 1.2.4.1. This technique has been used to identify the prevalence of the sarcopenic obesity phenotype in cancer outpatients (Prado et al., 2008, Tan et al., 2009) and abnormal body composition was shown to be related to increased toxicity for chemotherapy (Prado et al., 2007). However, cross-sectional CT at L3 level does not inform on limb muscle groups which could be used to correlate with measures of function of that particular muscle group and thus give a measure of muscle quality. In another study quadriceps muscle CSA was measured using MR imaging and muscle strength was measured using dynamometry in weight losing gastrointestinal cancer patients to infer change in mechanical muscle quality, but no attempt was

made to take account of fatty infiltration (Weber et al., 2009).

The aim of the study in this Chapter is to quantify contractile and non-contractile compartments within the knee extensor muscle group on MR images obtained from a small group of patients with cancer cachexia. While fatty infiltration may be readily visualized on MR images (Figure 6.1), quantification of its extent is challenging. In this preliminary study the application of a statistical technique, known as k-means clustering (JB, 1967), was used to quantify contractile (skeletal muscle) and non-contractile (including fat) components of the total quadriceps muscle mass.

The methodology for image analysis was developed by Dr Calum Gray, Dr Carolyn Greig and Dr Tom MacGillivray (University of Edinburgh) primarily to investigate its use in healthy older adults. Although it is the investigation of patients with cancer cachexia that form part of this thesis, the results from the healthy young and very elderly women will also be included in order to give broader context. In order to allow comparison with this healthy cohort, no male cancer patients were included in this study.

6.3 ***Hypotheses***

- Application of k-means statistical clustering would result in significant differences between MR imaging derived total quadriceps volume and quadriceps muscle (i.e. total volume minus non-contractile tissue),
- Cachectic muscle would contain a greater amount of fat compared with healthy young and elderly muscle
- The impact of k-means statistical clustering on muscle optimisation (i.e. separating the contractile from the non-contractile tissue component) would be greatest in cachectic muscle.

6.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

MR imaging and analysis (scan protocol, image optimisation, k-means statistical clustering analysis): see Section 2.6.1

Statistics: see Section 2.12.1

6.5 Results

Three groups of women participated in this study: young healthy, elderly healthy and newly diagnosed weight-losing UGI (pancreatic, oesophageal, gastric) cancer (Table 6.1).

The older healthy group were significantly older than either the cancer patients or the young healthy group ($p < 0.001$). There were no significant differences in body weight between the groups ($p = 0.086$). There was a trend towards a higher BMI in the cancer patients despite the patients having already lost an average of 8% of their pre-illness stable weight. The MR imaging procedure was well tolerated by all the participants.

Muscle volume

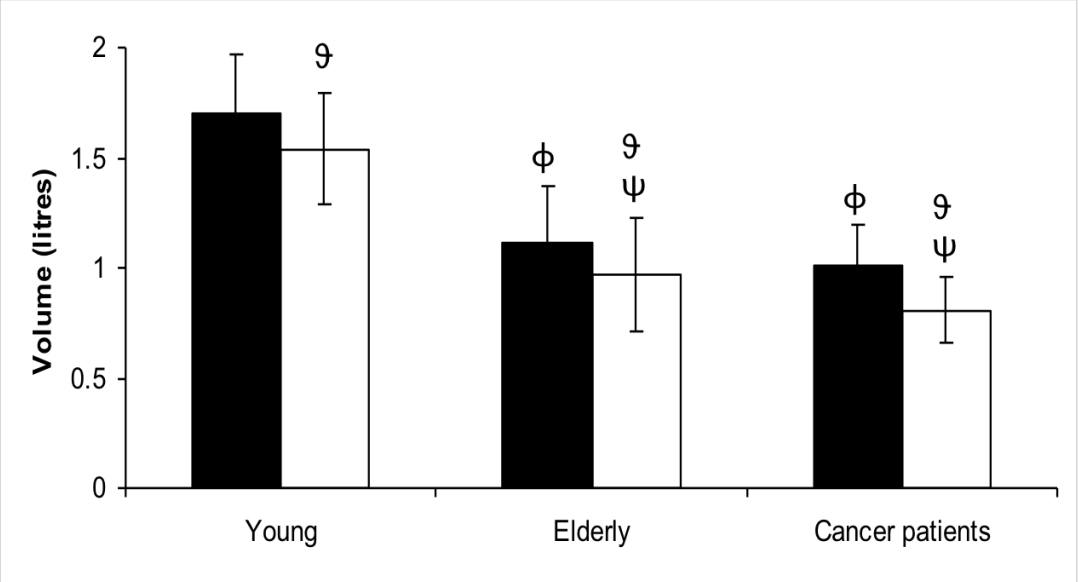
Figure 6.2 shows the data obtained for quadriceps volume both pre- and post- k-means clustering analysis (one leg). Pre- k-means analysis of a dataset comprising predominantly left leg data $n = 29/34$ and $n = 5/34$ right leg data gave mean values of quadriceps muscle volume for young women of 1.70 (0.27) l (range 1.12–2.11 l) and old women 1.12 (0.26) l (range 0.85–1.76 l). The patients with UGI cancer had a mean volume of 1.09 (0.18) l (range 0.80–1.30 l). The differences between groups were significant (ANOVA $f = 28.24$; df (2, 31); $p < 0.001$). Analysis of the right leg gave similar results (ANOVA $f = 31.26$; df (2,31); $p < 0.001$).

Table 6.1: Patient demographics

	Young healthy (n=16)	**Old healthy** (n=9)	**UGI cancer** (n=9)
Age (years)	26 (19–30)	80 (76–82)	62 (44–78)
Weight (kg)	64.3 (8.7)	55.6 (8.0)	61.1 (10.7)
BMI (kg/m^2)	22.9 (2.5)	23.2 (3.2)	24.4 (4.7)
Weight-loss (%)	N/A	N/A	8.3 (15.2)

Results are reported as mean (SD) except for age, which is given as median (range). Abbreviations: UGI, upper gastrointestinal; BMI, body mass index; kg, kilograms; m, metres; N/A, not applicable.

FIGURE 6.2: EFFECT OF K-MEANS STATISTICAL CLUSTERING ON MUSCLE VOLUME



Quadriceps volume of the left leg was measured in n=29/34 participants. Pre-k-means values (i.e. including non-contractile tissue) are represented in black, and post-k-means (i.e. only contractile tissue) in white. ANOVA showed a significant group effect, (f = 31.69; df (2,31); p < 0.001) and a significant effect of k-means analysis (ϑ = p<0.001 vs. pre-k-means of corresponding group). Post-hoc tests with Bonferroni correction showed significant differences between young/old and young/patients (but not old/patients) both pre- (ϕ = p<0.001 vs. young pre-k-means) and post- (Ψ = p<0.001 vs young post-k-means) analysis. Abbreviations: ANOVA, univariate analysis of variance.

Effect of k-means analysis

The effect of k-means analysis (Figure 6.2) was to significantly reduce the mean muscle volume by 9% (from 1.70 to 1.54 l) in the young women, by 14% (from 1.12 to 0.97 l) in older women and by 20% in the patient group (from 1.09 to 0.81 l) (ANOVA $f = 477.67$; df (1, 31); $p < 0.001$). ANOVA also showed a significant interaction ($f = 3.87$; df (2, 31); $p = 0.032$), which was due to the greater difference between pre- and post-optimized volumes in the cancer patient group (ANOVA $f = 3.88$; df (2,31); $p = 0.031$). (Analysis of the right leg data gave similar results ANOVA $f = 4.01$; df (2,31); $p = 0.028$). A comparison of optimized volumes showed that healthy old women had 37% and women with cancer 48% less muscle than healthy young women. Furthermore, there was a significant effect of the k-means optimization with respect to group (i.e., cancer vs. healthy) when controlling for age as a covariate ($p = 0.003$).

6.6 Discussion

After initial segmentation and volume measurement, application of the k-means technique resulted in a significant reduction in 'muscle' volume in each group, showing that even in healthy participants the quadriceps contains a significant amount of non-lean tissue. The percentage of non-contractile tissue in young (9%) and older (14%) healthy women respectively is similar to results from previous studies using the pixel histogram technique (Kent-Braun et al., 2000, Macaluso et al., 2002). However fatty infiltration was significantly greater in the women with cancer (20%). Furthermore the influence of cancer on fatty infiltration was still significant when age is taken into account. This finding suggests that even at this relatively early stage of the disease the muscles of patients with UGI cancer are not only smaller (due to disuse as well as age and disease) but also less homogeneous.

The k-means clustering technique used in this study allows a non-invasive estimate of non-contractile tissue (i.e. fatty infiltration) from the MR images obtained, but it is unable to differentiate the relative proportions of intermuscular to intramuscular (including intramyocellular and extramyocellular) fatty tissue. Such an approach would require the use of MR Spectroscopy or histological/ultrastructural analysis of tissue biopsies, but there are few studies to date reporting the influence of cancer (Weber et al., 2009) or even ageing (Schick et al., 2002, Cree et al., 2004, Machann et al., 2005) on the precise distribution of skeletal muscular fat or its association with functional outcomes.

Advances in body composition analysis techniques have enabled the identification and characterization of clinically important phenotypes. This is essential because it allows more efficient stratification of patients within the cachexia spectrum. For example, using estimates of whole body muscle mass derived from muscle CSA measurements at L3 level it has been possible to assess the prevalence of sarcopenic obesity in cancer outpatients (Prado et al., 2008, Tan et al., 2009). This phenotype is a predictor of poor prognosis in both malignant and non-malignant disease (Tan et al., 2009, Prado et al., 2008, Honda et al., 2007). Measuring the proportion of contractile tissue in a muscle group also potentially enables characterization of different phenotypes, as well as conferring the ability to measure small but functionally important changes in muscle mass consequent to interventions to improve muscle mass. An additional advantage is that an accurate measure of 'true' muscle could be combined with measures of strength and power of the same muscle group, thus providing insight into not only the metabolic but also the mechanical consequences of cachexia. Measurements of strength or power per unit CSA or volume of the same muscle or muscle group could thus not only serve as an additional functional marker of frailty but also inform current debate on the influence of cachexia on muscle mechanical quality. Subsequent chapters will explore muscle quality in cancer patients.

This chapter has presented a small exploratory study in which a non-invasive optimization technique to objectively separate the contractile and non-contractile volume components from MR images has been shown to be useful in the assessment and phenotyping of cancer cachexia patients and very elderly people. When

controlling for age, fatty infiltration appears to be greater in the muscles of weight-losing patients with UGI cancer. The potential mechanisms underlying the differences in muscle composition and the functional consequences require further investigation and will be addressed in subsequent chapters.

Chapter 7: **Muscle mass, function and mechanical quality phenotype in human cancer cachexia**

7.1 ***Abstract***

Using MR scans in combination with direct measures of muscle function, this Chapter examines the relationship between cachexia, QoL and the mass/function/mechanical quality of lower limb skeletal muscle in gastrointestinal cancer patients. Patients with weight-loss ≥10% were classified as cachectic. Quadriceps strength and lower limb power were measured in 54 patients with gastrointestinal cancer (n=24 cachectic) and 18 controls. Quadriceps CSA was measured in 33/54 patients and in all controls using MR imaging. Muscle mechanical quality was defined as quadriceps strength/unit quadriceps CSA. QoL was assessed using the EORTC QLQ-C30. In male cachectic patients, quadriceps strength (p=0.003), lower limb power (p=0.026), quadriceps CSA (p=0.019), and muscle quality (p=0.008) were reduced compared with controls. In female cachectic patients, quadriceps strength (p=0.001) and muscle quality (p=0.001) were reduced compared with controls. Hand grip dynamometry (HGD) did not differ significantly across the groups in either males or females. Physical function (p=0.013) and fatigue (p=0.004) as reported from QoL scores were reduced in male cachectic compared with non-cachectic patients, but not in females. The results of this Chapter showed that muscle quality is reduced in both male and female cancer patients. The degree of impairment of lower limb muscle mass, quality and function and the impact on QoL varies with weight-loss and sex.

7.2 Introduction

Chapter 6 described the application of a novel assessment technique in the analysis of MR images. This chapter describes utilisation of this technique to investigate muscle mass phenotype along with an assessment of muscle function, mechanical quality and QoL in a larger cohort of patients (men and women) with gastrointestinal cancer and weight-loss.

Although muscle wasting is a hallmark of cancer cachexia, there are relatively few data describing regional loss of muscle mass or function in either male or female patients. Muscle loss may have a direct influence on patients' QoL as well as a significant impact on treatment tolerance (Prado et al., 2007) and is therefore an important potential therapeutic target.

Lower limb musculature (e.g. the quadriceps) is prone to wasting in patients undergoing prolonged bed rest (Pisot et al., 2008) and muscle mass correlates with strength in healthy adults (Maughan et al., 1983). Therefore, it might be anticipated that cancer patients with loss of muscle mass would have at least a proportionate loss of muscle strength/power. Moreover, if the mechanical quality, defined as quadriceps strength/unit quadriceps CSA, also known as relative strength of muscle were reduced, the reduction in function would be proportionately greater. Quadriceps strength was shown to be reduced in cachectic pancreatic cancer patients (Weber et al., 2009). However, when lower limb extensor strength was normalised to quadriceps CSA, there was no apparent reduction in muscle mechanical quality (Weber et al., 2009). In contrast, animal models have suggested that the process of

cachexia involves selective and early loss of key elements of the contractile structures within skeletal muscle which would likely impact on muscle mechanical quality (Acharyya et al., 2005). Moreover, ultrastructural analysis has demonstrated myofibrillar disarray, irregular muscle membranes and dystrophic muscle morphology in the muscles of tumour-bearing cachectic animals (Acharyya et al., 2005).

Knowledge of the relationship between muscle mass and function and their potential influence on QoL in cancer cachexia is limited. The aim of the present study was to characterise regional changes in muscle function in gastrointestinal cancer patients with cachexia.

7.3 ***Hypotheses***

- Cancer patients (male and female) with cachexia would have poorer muscle strength/function than non-cachectic/low weight-loss cancer patients or healthy controls
- Poorer muscle function would impact on QoL in cancer patients with cachexia
- The response of upper and lower limb muscle compartments would differ to the impact of cachexia

7.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

Blood collection: see Section 2.8.1

Blood measures (CRP, albumin, haemoglobin, TT, cFT, oestradiol, LH, FSH, SHBG): see Section 2.9

Muscle function (IKES, LLEP, HGD): see Section 2.3

MR imaging and analysis: see Section 2.6.1

Muscle mechanical quality: see Section 2.7

QoL (EORTC QLQ-C30, KPS): see Section 2.4

Statistics: see Section 2.12.1

7.5 **Results**

Demographics: The healthy controls were older than the cancer patients (78 vs. 65 years, $p<0.001$), although the average age of non-cachectic and cachectic patients was similar in both males (mean (range): 68(43-88) years vs. 63(39-76) years, respectively, $p=0.130$) and females (65(44-76) years vs. 63(44-83) years, respectively, $p=0.791$). The average weight-loss of cancer patients was 8.4% and all healthy controls were weight stable. The distribution in site of primary tumour was similar between non-cachectic and cachectic patients (Chi-squared $p=0.438$), and in males and females (Chi-squared $p=0.195$) (Table 7.1). Likewise, there were no significant differences in distribution of tumour stage between groups (Table 7.2).

Blood measures/gonadal status: In males, albumin ($p<0.001$) and haemoglobin ($p=0.001$) differed significantly across the three groups (Table 7.2A). Post-hoc comparisons indicated that controls had significantly higher albumin levels than both the non-cachectic ($p<0.001$) and the cachectic patients ($p<0.001$). However, there was no significant difference in albumin levels between cancer patient groups ($p=0.519$). Control haemoglobin levels were significantly higher than those of both the non-cachectic ($p=0.002$) and the cachectic patients ($p=0.001$), but again there was no significant difference between cancer patient groups ($p=0.904$). While total testosterone levels (Table 7.3A) did not differ significantly in males ($p=0.801$), cFT levels were significantly different ($p=0.030$). Post-hoc comparisons showed a significantly higher cFT in controls compared with both the non-cachectic ($p=0.048$) and cachectic patients ($p=0.040$), but there was no significant difference in cFT level between male non-cachectic and cachectic patients ($p=0.931$).

Table 7.1: Distribution of patient numbers according to tumour site.

	Male cancer patients		Female cancer patients	
Site	Non-cachectic	Cachectic	Non-cachectic	Cachectic
Oesophageal	9	5	2	1
OGJ	3	1	1	0
Gastric	2	2	2	3
Pancreatic	6	6	5	4
Bile duct	0	1	0	0
Rectal	0	0	0	1

Values refer to number of patients in each group. Chi-squared: male vs. female, p=0.195; non-cachectic vs. cachectic cancer patients, p=0.438. Abbreviations: OGJ, oesophago-gastric junction.

Table 7.2: Demographics, blood profile, QoL scores and quadriceps strength, lower limb power and upper limb dynamometry in controls and cancer patients (with and without cachexia).

A. MALES

		Control	**Cancer patients**		**p value**	**(dof) F**
			Non-cachectic	Cachectic		
		(n=9)	(n=20)	(n=15)		
Age (years)		78 (3)	68(11)[A]	63(10)[A]	**0.002**	(2,41) 7.5
Height (cm)		171(4)	173(6)	175(7)	0.313	(2,41) 1.2
Weight (kg)		79.6 (9.9)	80.2(16.3)	73.8(11.3)	0.363	(2,41) 1.0
BMI (kg/m^2)		27.1(3.9)	26.8(5.1)	24.2(3.6)	0.158	(2,41) 1.9
Weight-loss (%)		0 (0)	1.5(3.0)	14.2(4.2)[A, B]	**<0.001**	(2,41) 83.7
MAMC (cm)		26.7(2.3)	25.7(2.7)	25.3(1.8)	0.397	(2,38) 0.9
Tumour Stage	1	-	1	0	0.846	-
	2	-	3	2		
	3	-	11	9		
	4	-	5	4		
Albumin (g/l)		43.6(2.7)	36.4(4.0)[A]	34.9(4.3)[A]	**<0.001**	(2,41) 14.9
CRP (mg/l)		4.3(2.8)	19.2(42.2)	19.6(28.8)[A]	0.723	(2,41) 0.3
Haemoglobin (g/l)		147.9(11.5)	125.2(17.4)[A]	122.9(13.7)[A]	**0.001**	(2,41) 8.8
KPS		100(0)	84(11)[A]	79(14)[A]	**<0.001**	(2,41) 10.3
QoL (global)		-	138(40)	86(58)	**0.006**	**-**
PF score		-	83(21)	60(29)	**0.013**	-
FA score		-	27(23)	56(32)	**0.004**	**-**
IKES (N)		391(61)	288(90)[A]	243(90)[A]	**0.001**	(2,38) 8.2
IKES (N/kg)		5.0(0.9)	3.7(1.3)[A]	3.2(1.0)[A]	**0.003**	(2,38) 6.6
LLEP (W)		157(30)	123(58)	98(45)[A]	**0.027**	(2,40) 3.9
LLEP (W/kg)		2.0(0.5)	1.5(0.5)	1.3(0.7)[A]	**0.033**	(2,40) 3.7
HGD (kg)		41(6)	38(10)	38(6)	0.593	(2,40) 0.5

B. FEMALES

		Control	Cancer patients		p value	(dof) F
			Non-cachectic	Cachectic		
		(n=9)	(n=10)	(n=9)		
Age (years)		79(3)	65(10)[A]	63(11)[A]	**0.001**	(2,25) 9.6
Height (cm)		159(6)	161(6)	160(8)	0.838	(2,25) 0.2
Weight (kg)		61.5(8.2)	66.4(11.8)	58.9(9.6)	0.276	(2,25) 1.4
BMI (kg/m^2)		24.4(3.8)	25.7(4.1)	22.9(2.4)	0.237	(2,25) 1.5
Weight-loss (%)		0 (0)	2.8(5.8)	20.5(10.4)[A, B]	**<0.001**	(2,25) 24.3
MAMC (cm)		21.1(2.1)	22.1(1.6)	21.9(1.4)	0.457	(2,22) 0.8
Tumour Stage	1	-	2	2	0.541	-
	2	-	2	0		
	3	-	4	4		
	4	-	2	3		
Albumin (g/l)		43.4(2.5)	35.4(5.3)[A]	31.2(4.6)[A]	**<0.001**	(2,25) 18.4
CRP (mg/l)		2.9(3.1)	9.7(24.4)	40.4(49.8)[A, B]	**0.008**	(2,25) 5.9
Haemoglobin (g/l)		132.0(7.8)	121.2(13.7)	109.6(19.5)[A]	**0.011**	(2,25) 5.4
KPS		100(0)	90(12)	77(16)[A, B]	**0.001**	(2,25) 9.1
QoL (global)		-	152(41)	100(55)	0.057	-
PF score		-	86(24)	73(24)	0.314	-
FA score		-	26(34)	44(39)	0.356	-
IKES (N)		296(75)	252(70)	159(68)[A, B]	**0.002**	(2,24) 8.6
IKES (N/kg)		4.8(1.1)	3.9(0.9)	2.7(1.1)[A]	**0.001**	(2,24) 9.2
LLEP (W)		67(28)	63(14)	59(20)	0.718	(2,24) 0.3
LLEP (W/kg)		1.1(0.4)	1.0(0.2)	1.0(0.3)	0.795	(2,24) 0.2
HGD (kg)		23(6)	27(10)	22(5)	0.314	(2,23) 1.2

Values are presented as mean (SD). F statistic is presented for one-way ANOVA comparisons. [A] p≤0.05 (Tukey's post-hoc) vs. control, [B] p≤0.05 (Tukey's post-hoc) non-cachectic vs. cachectic cancer patients. Abbreviations: dof, degrees of freedom; BMI, body mass index; MAMC, mid arm muscle circumference; CRP, C reactive protein; KPS, Karnofsky performance score; QoL, quality of life; PF, physical function; FA, fatigue; HGD, Hand grip dynamometry; IKES, Isometric knee extensor strength; LLEP, Lower limb explosive power; cm, centimetres; kg, kilograms; m, metres; g, grams; l, litres; mg, milligrams; N, newtons; W, watts.

Table 7.3: Gonadal status of controls and cancer patients (with and without cachexia).

A. MALES

	Control	**Cancer patients**		**p value**	**(dof) F**
		Non-cachectic	Cachectic		
	(n=9)	(n=20)	(n=12)		
TT(nmol/l)	15.2(4.3)	13.4(7.6)	13.9(5.5)	0.801	(2,38) 0.2
cFT(nmol/l)	0.262(0.063)	0.193(0.073)[A]	0.184(0.067)[A]	**0.030**	(2,34) 3.8
Oestradiol(pmol/l)	113.4(17.8)	85.0(35.6)	87.9(23.2)	0.074	(2,35) 2.8
LH(U/l)	4.2(2.2)	10.7(8.3)	10.4(5.9)	0.073	(2,37) 2.8
FSH(U/l)	5.8(3.3)	11.8(9.6)	13.3(10.8)	0.166	(2,38) 1.9
SHBG(nmol/l)	44.0(15.2)	59.9(42.4)	69.0(32.3)	0.285	(2,38) 1.3

B. FEMALES

	Control	**Cancer patients**		**p value**	**(dof) F**
		Non-cachectic	Cachectic		
	(n=8)	(n=8)	(n=7)		
TT(nmol/l)	1.4(0.5)	1.4(0.6)	0.9(0.3)	0.084	(2,20) 2.8
Oestradiol(pmol/l)	61.0(21.4)	50.6(25.2)	51.6(28.2)	0.692	(2,19) 0.4
LH(U/l)	25.1(8.3)	31.5(18.2)	20.4(12.5)	0.304	(2,20) 1.3
FSH(U/l)	66.3(23.3)	68.8(40.4)	32.4(20.9)	0.054	(2,20) 3.4
SHBG(nmol/l)	63.7(25.6)	64.8(18.4)	60.2(33.3)	0.949	(2,17) 0.1

Values are presented as mean (SD). F statistic is presented for one-way ANOVA comparisons. [A]p≤0.05 (Tukey's post hoc) vs. control. Abbreviations: dof, degrees of freedom; cFT, calculated free testosterone; TT, total testosterone; LH, luteinising hormone; FSH, follicle stimulating hormone; SHBG, sex hormone binding globulin; nmol, nanomoles; l, litres; U, units; pmol, picomoles.

In females, albumin ($p<0.001$), haemoglobin ($p=0.011$) and CRP ($p=0.008$) differed significantly across the three groups (Table 7.2B). Post-hoc comparisons indicated that controls had significantly higher albumin levels than both the non-cachectic ($p=0.001$) and the cachectic patients ($p<0.001$), but that there was no significant difference between cancer patient groups ($p=0.112$). The haemoglobin levels of the control group were significantly higher than the cachectic patients ($p=0.008$), but not the non-cachectic patients ($p=0.253$). There was no significant difference in haemoglobin levels between female cancer patient groups ($p=0.205$). CRP levels in cachectic patients were significantly higher than in the controls ($p=0.018$) and non-cachectic patient groups ($p=0.018$). CRP was similar in controls and non-cachectic patients ($p=0.987$). Measures of hormonal status in females did not differ significantly across the groups (Table 7.3B), although female cachectic patients did show a trend towards lower FSH levels ($p=0.054$).

Upper and lower limb muscle strength/power: Table 7.2 shows the HGD, IKES and LLEP data. HGD did not differ significantly across the groups for either males ($p=0.593$) or females ($p=0.314$). However, in males, there were significant differences across the groups for IKES ($p=0.003$) and LLEP ($p=0.033$). Post-hoc comparisons showed control males to have higher IKES than both non-cachectic ($p=0.022$) and cachectic patients ($p=0.003$). Comparison of IKES between non-cachectic and cachectic patients was not significant ($p=0.480$). Cachectic patients had significantly lower LLEP than controls ($p=0.026$). There were no significant differences in LLEP between control and non-cachectic patients ($p=0.123$), nor between non-cachectic and cachectic patients ($p=0.576$).

There was a significant difference across the female groups for IKES (p=0.001), but not for LLEP (p=0.795). Post-hoc comparisons showed IKES to be significantly lower in cachectic patients compared with controls (p=0.001), and a trend towards being reduced compared with non-cachectic patients (p=0.069). IKES in controls was not significantly different from non-cachectic patients (p=0.151).

Quadriceps muscle CSA and degree of fat infiltration: Quadriceps CSA was measured in a subgroup of cancer patients (33/54; 61%). The remaining 21 patients were not assessed due either to patient preference or for logistical reasons. The demographics of the sub-group did not differ from the overall cancer patient cohort (Table 7.4).

In males, there was a significant difference in quadriceps CSA across the groups (p=0.012) (Table 7.5). Post-hoc comparisons showed cachectic patients to have significantly smaller quadriceps CSA than both control (p=0.049) and non-cachectic patients (p=0.019). Controls and non-cachectic patients had similar quadriceps CSA (p=0.996). In females, there was no significant difference in quadriceps CSA across the groups (p=0.159) (Table 7.5). Although mean percentage fatty infiltration was higher in the cancer patients (particularly the non-cachectic patients) than controls, the results did not differ significantly across the groups for either sex (p=0.412 males and p=0.149 females).

Table 7.4: Demographics of cancer patient MR imaging subgroup versus all cancer patients.

		Male cancer patients		Female cancer patients	
		All (n=35)	MR cohort (n=17)	All (n=19)	MR cohort (n=16)
Age (years)		66 (11)	64 (12)	64 (10)	65 (10)
Height (cm)		174 (7)	175 (7)	160 (7)	159 (7)
Weight (kg)		77.5 (14.6)	77.5 (16.6)	62.9 (11.2)	60.4 (9.2)
BMI (kg/m^2)		25.7 (4.6)	25.6 (5.5)	24.4 (3.6)	23.9 (3.4)
Weight-loss (%)		7.0 (7.3)	10.7 (7.6)	11.2 (12.1)	11.6 (13.0)
MAMC (cm)		25.5 (2.4)	25.8 (2.4)	22.0 (1.5)	21.8 (1.4)
Tumour Stage	1	1	0	4	4
	2	5	1	2	2
	3	20	10	8	5
	4	9	6	5	5

Values are presented as mean (SD). Comparison of means between all cancer patients and the MR imaging cohort for both males and females (Student's t-test) revealed no statistically significant differences. MR, magnetic resonance; BMI, body mass index; MAMC, mid arm muscle circumference; cm, centimetres; kg, kilograms; m, metres.

Table 7.5: Quadriceps muscle mass, mechanical quality and fatty infiltration status of controls and cancer patients (with and without cachexia).

A. MALES

	Control	**Cancer patients**		**p value**	**(dof) F**
		Non-cachectic	Cachectic		
	(n=9)	(n=6)	(n=11)		
Q. CSA (mm^2)	6553(663)	6617(1652)	5666(805)	0.098	(2,23) 2.6
Q. CSA/height^2(mm^2/m^2)	2236(263)	2222(466)	1823(247)$^{A, B}$	**0.012**	(2,23) 5.4
Fat free Q. CSA (mm^2)	5665(644)	5246(1983)	4842(1080)	0.339	(2,21) 1.1
Fat infiltration (%)	13.4(6.6)	20.3(12.2)	15.1(9.6)	0.412	(2,21) 0.9
Muscle Quality (N/mm^2)	6.0(1.2)	4.4(0.8)	4.0(1.5)^{A}	**0.008**	(2,21) 6.1

B. FEMALES

	Control	**Cancer patients**		**p value**	**(dof) F**
		Non-cachectic	Cachectic		
	(n=9)	(n=8)	(n=8)		
Q. CSA (mm^2)	4613(680)	4152(471)	4152(725)	0.244	(2,22) 1.5
Q. CSA/height^2(mm^2/m^2)	1831(252)	1660(226)	1622(208)	0.159	(2,22) 2.0
Fat free Q. CSA (mm^2)	4105(655)	3491(542)	3560(790)	0.137	(2,22) 2.2
Fat infiltration (%)	11.1(3.1)	16.1(7.2)	14.9(5.1)	0.149	(2,22) 2.1
Muscle Quality (N/mm^2)	6.4(1.0)	5.7(1.7)	3.6(1.4)$^{A, B}$	**0.002**	(2,21) 9.0

Values are presented as mean (SD). F statistic is presented for one-way ANOVA comparisons. ^{A}p≤0.05 (Tukey's post-hoc) vs. control, ^{B}p≤0.05 (Tukey's post-hoc) non-cachectic vs. cachectic cancer patients. Abbreviations: dof, degrees of freedom; Q. CSA, Mid-femur quadriceps cross-sectional area; mm, millimetres; m, metres; N, newtons.

Quadriceps muscle mechanical quality: Quadriceps muscle mechanical quality (N/mm^2) correlated negatively with % weight-loss in both males (R= -0.48, p=0.018) and females (R= -0.63, p=0.001). There was a significant difference in muscle mechanical quality across the three groups in both males (p=0.008) and females (p=0.002) (Table 7.5). Post-hoc comparisons demonstrated that male cachectic patients had lower muscle quality than controls (p=0.008). There was also a trend towards muscle quality being lower in male non-cachectic patients compared with controls (p=0.079). There were no significant differences in muscle mechanical quality between male cancer patient groups (p=0.870).

In females, muscle mechanical quality was significantly lower in cachectic patients compared with both controls (p=0.023) and non-cachectic patients (p=0.001), but no significant difference was detected between control and non-cachectic patients (p=0.578) (Table 7.5).

Influence of systemic inflammation on muscle strength/power and mechanical quality: In males, CRP correlated negatively with IKES (R= -0.42, p=0.006) and LLEP (R= -0.38, p=0.012), but not with muscle quality (R= -0.33, p=0.120). A univariate ANOVA model of IKES was constructed incorporating age, tumour stage and CRP (R squared, 0.25). Only CRP was a significant determinant (p=0.019, effect size 18.2%).

In females, CRP correlated negatively with IKES (R= -0.60, p=0.001) but not with LLEP (R= -0.19, p=0.341). However, there was a significant negative correlation with muscle quality (R= -0.59, p=0.003). Again, univariate ANOVA modelling (R

squared, 0.41) demonstrated CRP as the only significant determinant of IKES ($p=0.010$, effect size 38.8%).

QoL and performance scores: KPS scores were significantly different across the groups for both males ($p<0.001$) and females ($p=0.001$) (Table 7.1). Post-hoc comparisons revealed that, in males, controls had higher KPS than both non-cachectic ($p=0.003$) and cachectic patients ($p<0.001$). There were no significant differences in KPS between cancer patient groups ($p=0.361$). In females, cachectic patients had lower KPS than controls ($p=0.001$) and non-cachectic patients ($p=0.050$). No significant difference in KPS was shown between control and non-cachectic patients ($p=0.169$).

Global QoL scores (EORTC QLQ C-30) correlated negatively with % weight-loss in male cancer patients ($R= -0.52$, $p=0.003$), but this association was absent in female cancer patients ($R= -0.29$, $p=0.292$). In males, physical function score (PF2 component) was significantly lowered in cachectic compared with non-cachectic patients ($p=0.013$). The fatigue score (FA component) was significantly higher in cachectic compared with non-cachectic male patients ($p=0.004$). Conversely, in cachectic female patients, neither the physical function score ($p=0.314$) nor the fatigue score ($p=0.356$) were significantly different compared with non-cachectic patients. In male cancer patients, IKES and mechanical quality correlated significantly with global QoL ($R= 0.38$, $p=0.043$ and $R= 0.58$, $p=0.031$ respectively) and physical function score ($R= 0.41$, $p=0.028$ and $R= 0.64$, $p=0.014$ respectively). Quadriceps CSA did not correlate significantly with any subjective QoL variables.

In females, there were no significant correlations between subjective QoL variables and IKES, CSA and mechanical quality. There was a trend towards a positive correlation between global QoL and mechanical quality ($R= 0.52$, $p=0.068$) and between physical function score and quadriceps CSA ($R= 0.52$, $p=0.066$).

7.6 ***Discussion***

This study demonstrates that in gastrointestinal cancer patients there is variability in lower limb muscle function, mechanical quality and mass according to the degree of weight-loss and sex. While lower limb muscle mass, strength and power declined in male cancer patients, females appeared to experience attenuated loss of muscle mass and power. Mechanical quality was reduced in both male and female cancer patients, but only in females did it decline progressively with cachexia. Such sexual dimorphism extended to the impact of cachexia on QoL: a significant association was shown between decreased quadriceps mechanical quality and a decline in subjective QoL measures in males, but not in females. These findings are consistent with recent literature suggesting sexual dimorphism may impact on the effects of systemic disease such as cirrhosis (Peng et al., 2007) and cancer (Palomares et al., 1996, Baracos et al., 2010). In a recent analysis of body composition based on CT, 61% of males presenting with non-small cell lung cancer were classified as sarcopenic compared with only 31% of females (Baracos et al., 2010). Another study of lung cancer patients demonstrated an eightfold faster rate of weight-loss in males and a reduced survival compared with females matched for disease stage (Palomares et al., 1996).

The healthy control group in the present study were approximately a decade older than the cancer patients. It is known that even healthy ageing results in gradual muscle atrophy and decline in muscle function (Thomas, 2007). That a significant impairment of muscle function and quality was demonstrated despite the HC

experiencing a further 10-year duration of age-associated muscle loss highlights the major impact of cancer. However, the age difference may have impacted on the results for the CSA and fatty infiltration. In Chapter 6, it was shown that female cancer patients had smaller and fattier quadriceps muscles than healthy young and older women. The healthy older women also had smaller and fattier muscles than the younger women indicating these are features of sarcopenia of ageing. If the controls in the current study had been exactly age matched, it is likely that there would have been a significant effect of cancer cachexia on the CSA for women as well as men, and also likely that percentage differences in fatty infiltration would have been more pronounced. It is interesting to see that for both men and women, the non-cachectic cancer patients had higher % fatty infiltration than the cachectic patients. Although this observation was not significantly different, it might suggest that fatty infiltration is an early phenomenon in the cachexia journey before there is an increase in adipose tissue loss with more advanced cachexia. Whether inter or intramuscular fat contributes to poorer muscle quality is plausible, but requires further investigation. This will be addressed in Chapter 8.

Consistent with the majority of cancer studies, cancer patients showed evidence of nutritional deficit (low albumin), anaemia (low haemoglobin) and systemic inflammation (raised CRP). However, other than for CRP in female patients, there did not appear to be a further significant deterioration in the presence of cachexia. This could indicate that changes in these blood parameters occur early in the cancer cachexia journey, or simply reflect a wide heterogeneity of response amongst the cancer patients.

Quadriceps CSA was similar in female non-cachectic and cachectic patients. One potential explanation would be that loss of adipose tissue alone (rather than in combination with lean tissue) accounted for the weight-loss in this group. A more likely explanation is that there was a small reduction in muscle mass that could not be detected due to the relatively small sample size. In contrast to the females, quadriceps CSA was reduced by 15% in males with cachexia compared with male non-cachectic patients. These findings are consistent with observations in lung cancer patients, where significant muscle wasting is twice as prevalent in males compared with females (Baracos et al., 2010). Indeed, weight-loss (including muscle loss) has been suggested to play a role in mediating sex-related differences in lung cancer patient survival (Palomares et al., 1996). Due to the relatively small cohorts of patients with oesophageal, gastric and pancreatic cancer in the present study, survival analysis was not possible. However it is interesting to note that other investigators have reported sex-specific differences in survival for similar cancer types (Skipworth et al., 2011, Oberaigner and Siebert, 2011).

It has been suggested by Weber et al that muscle mechanical quality (i.e. quadriceps strength/ CSA) may be preserved in cancer cachexia (Weber et al., 2009). However, this previous study examined a relatively small number of patients with a single tumour type (pancreatic cancer), combined males and females within cohorts, and compared controls with cachectic patients at one extreme of the weight-loss spectrum. In contrast, the present study is larger, includes different tumour types and analyses males and females separately. Furthermore, the inclusion of controls and cancer

patients with varying degrees of weight-loss allowed differentiation between the impact of cancer alone on muscle function/quality and any additional effect of cachexia. Our findings parallel the reduced muscle quality observed in other human models of muscle atrophy such as old age (Goodpaster et al., 2006) and are consistent with the concept that reduced muscle quality may represent a general response of human muscle to chronic wasting.

The mechanism underlying a reduction in quadriceps MQ in patients with cancer cachexia may differ between the sexes. In the present study, in non-cachectic male patients, quadriceps CSA and strength were both reduced and MQ declined. Although quadriceps CSA declined further in the presence of cachexia, there was no corresponding further deterioration in quadriceps strength, and thus MQ did not fall further. In female cancer patients, quadriceps CSA was unaffected by the presence or absence of cachexia and therefore the observed reduction in MQ in the cachectic patients was entirely due to a significant decline in strength. Such sexual dimorphism has previously been observed in an immobilisation study in which, relative to the loss of muscle mass, females sustained a proportionally greater loss of strength than males (Yasuda et al., 2005).

Although quadriceps muscle strength was reduced in both males and females with cancer, there was an apparent preservation of lower limb power in females. In contrast with quadriceps muscle strength, lower limb power is a more complex measurement dependent not only upon the force generating capacity but also the contractile speed of several muscle groups (not just the quadriceps). The

heterogeneity of response of these different muscles or, conceivably, variation in the ratio of Type I/II fibres between men and women may account for the differences observed. There is also evidence in healthy males and females, that there are gender differences in muscle fatigability and recovery, along with neuromuscular activation (Hakkinen, 1993). The present study did not extend to investigation of these factors, and whether they are important in the context of cancer cachexia has yet to be investigated.

The preservation of upper limb (hand grip) strength but not lower limb strength in the present study suggests that different muscle groups may respond to systemic pro-cachectic signals differently. It is also possible that mechanical factors may play a role as cancer patients with weight-loss have reduced physical activity levels (Moses et al., 2004). In healthy males undergoing 35 days of bed-rest, postural muscles were more susceptible to atrophy than non-antigravity muscles (Pisot et al., 2008). Thus increased sitting or bed rest may account for some of the differential regional wasting in the cancer patients. In older adults, hand grip strength has previously been identified as a sensitive marker of sarcopenia, being significantly associated with poor mobility (Lauretani et al., 2003). However, the results of the present study in cancer patients appear to indicate that measurement of HGD alone will not inform a decline in load-bearing muscle function. This is a significant observation in relation to the concept that HGD is a suitable outcome measure for therapeutic intervention trials in cancer cachexia.

Diminished muscle strength and mechanical quality could be the result of several influences, including systemic factors such as anorexia (limiting energy/protein intake) as well as behaviour (poor motivation resulting in physical under-performance). In addition, factors intrinsic to muscle such as mechanical inefficiency (for example, disturbed muscle architecture or fatty infiltration) may play a role. Systemic circulating factors that could influence muscle mass and function include pro-inflammatory cytokines and sex hormones. Markers of systemic inflammation (e.g. CRP) in patients with cancer cachexia have been associated with a poorer prognosis, reduced function, poor nutrition and a shortened survival (Fearon et al., 2006). In the present study, circulating levels of CRP correlated negatively with strength in both male and female cancer patients. In addition, increased plasma CRP was associated with reduced lower limb power in male and poorer muscle quality in female cancer patients. Univariate analysis suggested that the impact of systemic inflammation on quadriceps muscle strength was greater in female (effect size 38.8%) than in male cancer patients (effect size 18.2%).

In the current study, assessment of gonadal status demonstrated a reduction in cFT in male cancer patients compared with male controls. Testosterone stimulates production of anti-inflammatory cytokines such as IL-10 and inhibits release of TNFα, IL-1 and IL-6 (Stephens et al., 2008). Lower testosterone levels could therefore lead to an increase in pro-inflammatory cytokines that could activate muscle proteolysis. Skipworth and co-workers (Skipworth et al., 2011) have demonstrated that hypogonadism (low total and/or free testosterone levels) is associated with poorer survival in male cancer patients. It is well established that low

testosterone levels impact on muscle mass and function and possibly QoL. Furthermore, testosterone replacement has been shown to prevent loss of muscle in ageing males (Allan et al., 2008). In females, oestrogen withdrawal at menopause is associated with an accelerated loss of muscle tissue with the greatest rate of loss occurring in the earliest postmenopausal years (Aloia et al., 1991). The females in the present study were post-menopausal and thus the influence of cancer on gonadal status and muscle mass may be weaker due to already low oestradiol levels.

In the present study, males with cancer cachexia reported poorer global QoL and physical function and increased fatigue. Poorer QoL measures were also associated with reduced muscle mechanical quality. However, the observed differences in females did not achieve statistical significance. This could be due to the preservation of LLEP and CSA in the female patients. Indeed, a recent study has shown a significant association between LLEP and SF-36 vitality score in patients after stroke (Lewis et al., 2011). However, given that the female cohort was smaller, it is also possible that the impact on QoL in females was masked by a Type II error.

In summary, the present study suggests that lower limb muscle mechanical quality is impaired in cancer patients. Furthermore, there appear to be sex differences in the impact of cachexia at the level of both muscle function and global functional status.

Chapter 8: **Muscle quality phenotype in human cancer cachexia: investigating intramyocellular lipid droplets**

8.1 ***Abstract***

The reason for the deterioration in muscle quality seen in Chapter 7 is not clear. There may be systemic factors or local muscle factors responsible. Chapter 6 showed that female cancer patients had increased fatty infiltration of quadriceps muscle. However, the contribution of inter versus intramuscular fat could not be determined. Such fat accumulation might be both a marker and mediator of metabolic change (e.g. insulin resistance) and muscle wasting. This Chapter describes a novel electron microscopy study to assess lipid droplets in cachectic cancer patients. Intramyocellular lipids are an important source of fuel for mitochondrial fat oxidation and play an important role in intramuscular lipid homeostasis. 19 UGI cancer patients and 6 healthy controls undergoing surgery were recruited. A Rectus abdominis biopsy was performed and processed for transmission electron microscopy (TEM). The number of intramyocellular lipid droplets and lipid droplet diameter were calculated from the TEM images. CT scans, performed as part of patients' routine care, were analysed to determine amount of adipose (intermuscular, visceral and subcutaneous) and muscle tissue. Compared with controls, UGI cancer patients had increased numbers of lipid droplets (mean (SD) 1.8 (1.9) vs. 6.4 (9.1) per 2650x field respectively, p=0.036). Mean lipid droplet diameter was also higher in UGI cancer patients compared with controls (0.42 (0.13) vs. 0.24 (0.21) μm, p=0.015). Mean lipid droplet count correlated positively with the severity of weight-loss (R= 0.51, p=0.025) and negatively with CT-derived measures of intermuscular fat (R= -0.53, p=0.022) and visceral fat (R= -0.51, p=0.029). This study suggests that the number and size of intramyocellular lipid droplets is increased in the presence of cancer and increases further with weight-loss/loss of adipose mass in other body compartments. The specific mechanisms and drivers of this phenomenon remain to be elucidated, but could relate to enhanced lipolysis or mitochondrial dysfunction in skeletal muscle.

8.2 Introduction

Chapter 7 demonstrated that weight-loss and gender impact on muscle function and mass in cancer cachexia patients. Furthermore, there was a reduction in muscle mechanical quality in male and female cancer patients compared with controls. The reasons for these changes are not clear. Systemic factors may contribute such as anorexia with resultant reduced protein/energy intake, impact of the systemic inflammatory response along with behavioural factors such as poor motivation and physical inactivity. Local factors could also play a role within muscle itself due to mechanical or biochemical inefficiency. In Chapter 6 and 7, there was evidence of increased fatty infiltration on MR imaging of the quadriceps. However, as highlighted in Chapter 6, the proportion of intra versus intermuscular fat could not be determined using the k-means clustering technique, and thus the relevance of intramyocellular fat in determining muscle quality in cancer cachexia is unknown. One study using MR spectroscopy showed increased intramyocellular lipid levels in cachectic patients (Weber et al., 2009) and it may be that this influences muscle quality/function. Thus, in order to explore this further, this chapter investigates the relationship between intramyocellular fat (lipid droplets) and cancer cachexia in a small series of muscle biopsies using transmission electron microscopy (TEM).

Whilst both lean and adipose tissue become depleted in cancer cachexia, there is evidence that body fat is lost more rapidly than lean tissue (Fouladiun et al., 2005). The drivers of lipolysis, or possibly reduced lipogenesis (Tisdale, 2009), in cancer cachexia are still to be elucidated fully. However TNFα (Plomgaard et al., 2008),

ZAG (Bing et al., 2004, Mracek et al., 2011) and MIC-1 (Ding et al., 2009) have been suggested as potential mediators. Adipose tissue is composed predominately of stored lipid droplets (Finn and Dice, 2006) and is intimately involved with energy homeostasis and metabolism through secreted adipokines (Wang et al., 2008). Additionally, it influences insulin sensitivity, affects immune and inflammatory pathways and interacts with catecholamines (Balisteri CR, 2010, Lafontan, 2008). The catabolism of lipids generates fatty acids that can either be utilised by skeletal muscle or further metabolised to take part in the Krebs cycle (Finn and Dice, 2006). Triglyceride-containing lipid droplets are dynamic organelles stored on demand in all cells and grow through a fusion process mediated by SNARE proteins, including SNAP23 (Jagerstrom et al., 2009). Within skeletal muscle, it is thought that intramyocellular lipid/lipid droplets act as fuel stores for mitochondrial fat oxidation (Schrauwen-Hinderling VB, 2006). Lipid droplets are usually in direct contact with mitochondria presumably to allow rapid transport when required in situations such as exercise (Schrauwen-Hinderling VB, 2006). Indeed, intramyocellular lipid decreases upon acute exercise (Krssak et al., 2000, Decombaz et al., 2001, Rico-Sanz et al., 2000, Shaw et al., 2010) and almost completely disappears after marathon running (Staron et al., 1989, Kayar et al., 1986). Conversely, physical inactivity and a diet excessive in fat can lead to an increase in intramyocellular lipid (Shaw et al., 2010). Thus, it would appear that a 'static' rather than 'dynamic' pool of intramyocellular lipid is pathophysiological. Endurance training causes a rise in intramyocellular lipid content supporting the role of lipid droplets as an energy source during physical activity (Schrauwen-Hinderling VB, 2006). Although the presence of lipid droplets in skeletal muscle is part of the normal physiology of healthy individuals with or

without physical training, associations have been shown between increased droplet number and pathological states such as the presence of type 2 diabetes/insulin resistance (Schrauwen-Hinderling VB, 2006, Bostrom et al., 2009) and aging (Crane et al., 2010). With aging, not only are numbers of lipid droplets increased, but their association with mitochondria appears to be disrupted (Crane et al., 2010) and mitochondrial function is altered (Conley et al., 2000, Trounce et al., 1989). In the morbidly obese, raised intramyocellular lipid content has been reported to be associated with insulin resistance and to decrease after weight-loss/bariatric surgery (Gray et al., 2003, Greco et al., 2002). In patients with gastrointestinal cancer, increased levels of intramyocellular lipid have also been reported. Using MR spectroscopy, a 35% higher level of intramyocellular lipids was observed in patients with cachexia (defined by >10% weight-loss in previous 6 months) compared with weight stable cancer patients (Weber et al., 2009).

The phenotype associated with cancer cachexia (lipid mobilisation, insulin resistance (Tisdale, 2009), systemic inflammation (Stephens et al., 2008), sarcopenia (Tan et al., 2009) and reduced physical activity (Moses et al., 2004)) suggest that there could be a relationship between increasing weight-loss and the number/size of intramyocellular lipid droplets. The study in this chapter sought to carry out a quantitative morphological examination of intramyocellular lipid droplets in human cancer-associated weight-loss using TEM.

8.3 ***Hypotheses***

- Due to the phenotype associated with cancer cachexia there would be an association between increasing weight-loss and the number/size of intramyocellular lipid droplets.
- There would be a relationship between increased intramyocellular lipid droplets, and indices of whole body, visceral or muscle content of fat.

8.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

Muscle biopsy (*Rectus abdominis*) and blood collection: see Section 2.8.1

Blood measures (CRP, albumin, insulin, cortisol, glucose, HOMA-IR): see Section 2.9

TEM (sample preparation and image analysis): see Section 2.11

CT imaging and analysis: see Section 2.6.2

QoL (KPS): see Section 2.4

Statistics: see Section 2.12.1

8.5 ***Results***

Nineteen cancer patients (11 men, 8 women) and 6 controls (2 men, 4 women) were recruited. Demographics are shown in Table 8.1. There were 8 patients with pancreatic cancer, 4 gastric, 3 oesophageal, 3 oesophago-gastric junction and 1 patient with small bowel cancer. The mean BMI of both the cancer patient and control groups was >25 kg/m^2. Cancer patients were older than controls (mean (SD) age 67 (10) vs. 53 (8) years respectively, p=0.005) and had a significantly higher percentage weight-loss (6.0 (7.1) vs. -0.3 (1.4) % respectively, p=0.002). MAMC and arm muscle CSA were not significantly different between controls and cancer patients. There was a trend towards higher KPS in controls compared with cancer patients (98.3 (3.1) vs. 91.7 (8.6), p=0.083). Significantly more cancer patients exhibited systemic inflammation (CRP ≥5mg/l) than controls (n= 11/19 vs. 0/6 respectively, p=0.020 Fisher's exact test). Both the average lipid droplet number (6.4 (9.1) vs. 1.8 (1.9), p=0.036, see Figure 8.1) and the average lipid droplet diameter (0.42 (0.13) vs. 0.24 (0.21) μm, p=0.015) were higher in the cancer cohort compared with controls.

Changes associated with increasing weight-loss/altered body composition in the cancer patients

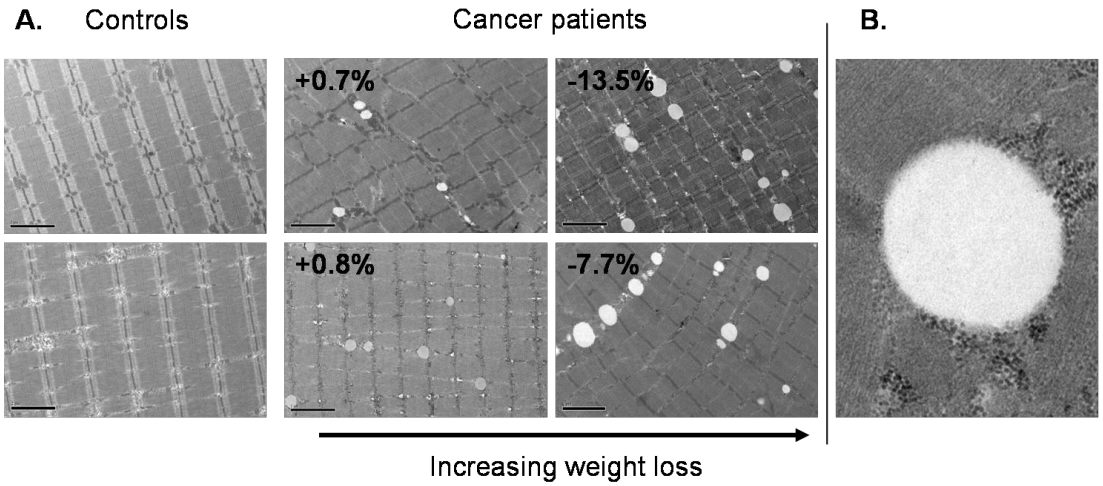
In the cancer patients, % weight-loss (range -10.4% to 17.1%) correlated negatively with MAC (R= -0.51, p=0.024) and MAMC (R= -0.46, p=0.047) and there was a trend towards a reduction in arm muscle CSA (R= -0.44, p=0.058). There were, however, no significant correlations between % weight-loss and CT-derived values of muscle mass. CT-derived measures of intermuscular and visceral fat were not

Table 8.1: Demographics, anthropometry, CT-derived body composition (cancer patients only), performance status, blood measurements, and intramyocellular lipid droplet count and size for cancer patients and controls.

		Control (n=6)	**Cancer** (n=19)	**p value**
M/F		2/4	11/8	0.378
Age (years)		53(8)	67(10)	**0.005**
Weight (kg)		68.3(12.3)	70.7(12.8)	0.684
BMI (kg/m^2)		26.0(4.5)	25.1(4.4)	0.671
Weight-loss (%)		-0.3(1.4)	6.0(7.1)	**0.002**
MAC (cm)		28.5(3.1)	28.8(3.0)	0.793
TSF (mm)		18.0(6.1)	14.6(7.4)	0.333
MAMC (cm)		22.8(3.0)	24.2(2.5)	0.259
Arm muscle CSA (cm^2)		34.3(9.4)	38.7(9.5)	0.339
KPS		98.3(4.1)	91.7(8.6)	0.083
Tumour stage	1	-	0	-
	2	-	6	-
	3	-	8	-
	4	-	4	-
CRP (mg/l)		2.1(1.4)	20.8(42.1)	0.295
SI/no SI		0/6	11/19	**0.020**
Albumin (g/l)*		36.6(6.0)	33.7(4.6)	0.248
Insulin (mU/l)*		-	5.8(3.2)	-
Glucose (mmol/l)*		-	6.2(1.6)	
Cortisol (nmol/l)*		-	454(109)	-
HOMA-IR*		-	1.8(0.9)	-
Cortisol:Insulin ratio*		-	96.6(48.1)	-
IM fat (cm^2/m^2)		-	11.0(5.9)	-
Vi fat (cm^2/m^2)		-	115.2(95.3)	-
SC fat (cm^2/m^2)		-	161.1(66.4)	-
Skeletal muscle (cm^2/m^2)		-	43.1(7.6)	-
LD count		1.8(1.9)	6.4(9.1)	**0.036**
LD diameter (μm)		0.24(0.21)	0.42(0.13)	**0.015**

*Results are presented as mean (SD). Comparison between groups was made using Student's t-test/Fisher's exact test. SI was defined as CRP ≥5mg/l. *Due to sample limitations, not all patients had these variables measured: Albumin n=18, Insulin n=13, Glucose n=14, Cortisol n=17, HOMA-IR n=10, Cortisol:Insulin ratio n=17. Abbreviations: BMI, body mass index; CRP, C-reactive protein; CSA, cross-sectional area; HOMA-IR, homeostatic model assessment of insulin resistance; IM, intermuscular; KPS, Karnofsky performance score; LD, lipid droplet; MAC, mid arm circumference; MAMC, mid arm muscle circumference; SI, systemic inflammation; SC, subcutaneous; TSF, triceps skin fold thickness; Vi, Visceral; kg, kilograms; m, metres; cm, centimetres; mm, millimetres; mg, milligrams; g, grams; U, units; mU, milliUnits; mmol, millimoles; nmol, nanomoles; μm, micrometres; CT, computed tomography; M, male; F, female.*

FIGURE 8.1: REPRESENTATIVE ELECTRON MICROGRAPHS FOR CONTROLS AND CANCER PATIENTS.

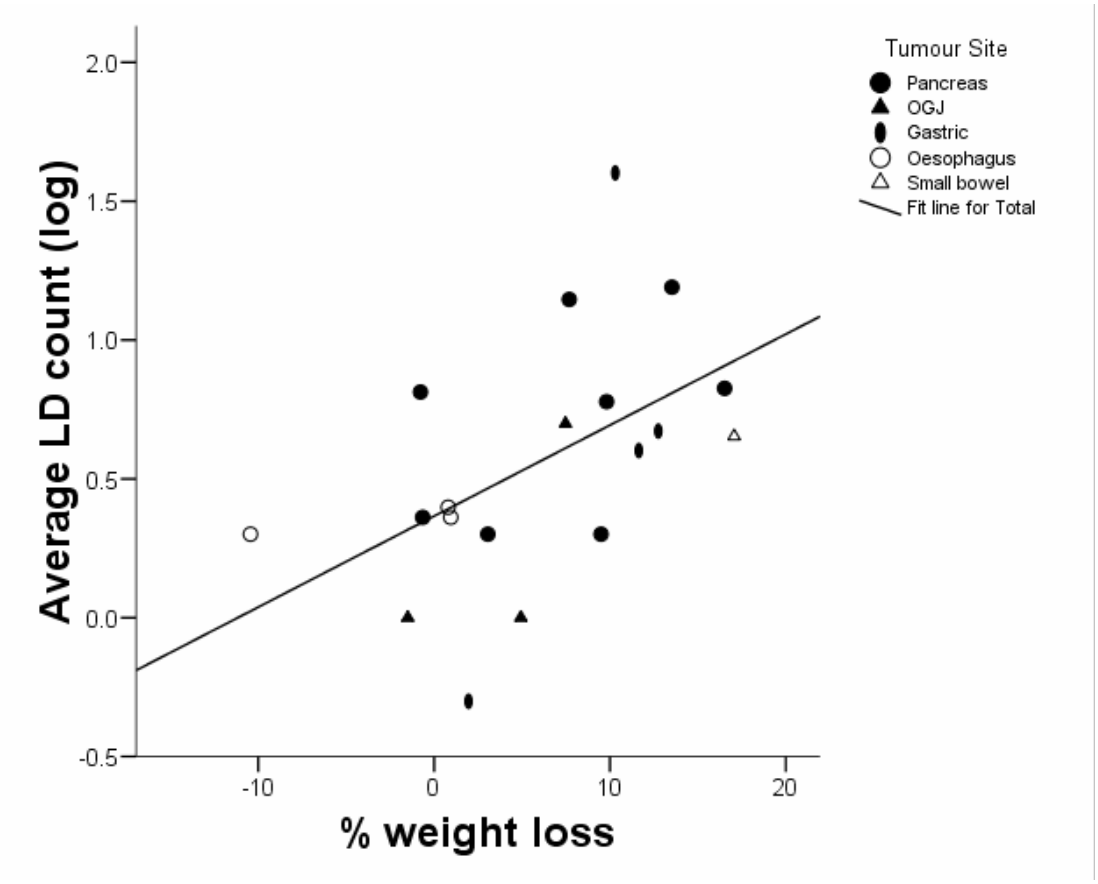


A. *Images are all at 2650x magnification. Greater numbers of lipid droplets (white vacuoles) were evident with increasing weight-loss. % values for cancer patients refer to weight change from pre-illness stable weight. Bars represent 2μm.*
B. *11000x magnification image of a single lipid droplet demonstrating the absence of a double membrane, thus distinguishing it from vacuolated giant mitochondria (Gdynia et al., 2010).*

significantly associated with weight-loss but there was a trend towards a (negative) association between weight-loss and subcutaneous fat (R= -0.42, p=0.082). Weight-loss correlated negatively with albumin (R= -0.56, p=0.015), but there were no significant associations with CRP (R= 0.15, p=0.549), insulin (R= -0.32, p=0.280), glucose (R= 0.19, p=0.509), cortisol (R= -0.27, p=0.295), HOMA-IR (R= -0.18, p=0.614) or cortisol:insulin ratio (R= 0.44, p=0.137).

The average lipid droplet number correlated positively with % weight-loss (R= 0.51, p=0.025, Figure 8.2), but there was no relationship between weight-loss and lipid droplet diameter (R= 0.15, p=0.535). MAMC, arm muscle CSA and CT-derived muscle mass did not correlate significantly with either lipid droplet count or lipid droplet diameter. Both weight (R= -0.61, p=0.007) and BMI (R= -0.46, p=0.050) correlated negatively with the number of lipid droplets. There were significantly greater numbers of lipid droplets in patients with lower CT-derived measures of intermuscular fat (R= -0.53, p=0.022) and visceral fat (R= -0.51, p=0.029) and there was also a trend towards an association between droplet number and subcutaneous fat (R= -0.46, p=0.055). No relationship was demonstrated between patients' age and number of lipid droplets (R= -0.30, p=0.209) or between the number of lipid droplets and the lipid droplet diameter (R= 0.34, p=0.152). There were no significant associations between the number of lipid droplets and plasma levels of albumin (R= -0.28, p=0.256), CRP (R= -0.10, p=0.695), insulin (R= -0.26, p=0.391), glucose (R= -0.03, p=0.926), cortisol (R= -0.13, p=0.633), HOMA-IR (R= -0.38, p=0.280) or cortisol:insulin ratio (R= 0.09, p=0.783).

FIGURE 8.2: WEIGHT-LOSS VERSUS LIPID DROPLET NUMBER



There was a significant positive correlation between weight-loss (%) and LD number (R= 0.51, p=0.025; Pearson's correlation, two-tailed). There was no significant difference in LD number according to tumour site (one-way ANOVA, p=0.559). Abbreviations: LD, lipid droplet; OGJ, oesophago-gastric junction.

8.6 ***Discussion***

The current study demonstrates a progressive rise in the number of intramyocellular lipid droplets in relation to both increasing weight-loss and depletion of adipose tissue mass in patients with cancer. In addition, the presence of cancer was associated with increased lipid droplet numbers compared with controls. Thus, the presence of increased intramyocellular lipid droplets does not seem to be related exclusively to weight-loss but rather is probably linked to the mediators of the processes underlying such weight-loss (which is variably expressed depending on the individual patient). Increasing weight-loss, however, does seem to exacerbate the observed changes in ultrastructure.

Patients with obesity have increased intramyocellular lipid droplets (van Loon et al., 2004), whereas the current study observed a paradoxical negative association between BMI and number of lipid droplets: that is, the lower the BMI, the higher the number of lipid droplets. These findings suggest that increased intramyocellular lipid droplets are not specific to either a net positive or negative energy balance but may relate to the underlying causes of shifts in energy balance.

Consistent with the above hypothesis, we observed significant negative associations between CT-derived measures of fat mass (intermuscular, visceral and subcutaneous) and the number of intramyocellular lipid droplets. This would suggest that the changes within the myocytes were not related exclusively to local (skeletal muscle) events but rather to the overall state of net lipid mobilisation and negative energy balance or perhaps endocrine disruption.

The size of lipid droplets as well as their number may be physiologically significant. However, in the present study, although the average number of lipid droplets increased with progressive weight-loss, there was no association between weight-loss and lipid droplet diameter. This could represent the lipid droplets reaching a plateau of growth or may simply be due to there being a wide variability in lipid droplet diameter.

The cancer patients in the present study were overweight (mean BMI >25kg/m^2) and this probably reflects not only the increasing level of obesity in the general population, but that obesity is a risk factor for the development of cancer. It is important to recognise that underneath this mantle of fat there may be clinically occult muscle wasting. Indeed, in the present study there was an association between increasing weight-loss and reduced MAMC (an anthropometric index of whole body muscle mass). The trend towards sarcopenic obesity in advanced cancer patients has recently been emphasised (Prado et al., 2008). Indeed, a recent study of patients with advanced pancreatic cancer demonstrated sarcopenic obesity to be an independent determinant of reduced survival (Tan et al., 2009). The present study did not relate either anthropometric or CT-derived measures of muscle mass to the number of intramyocellular lipid droplets, but this probably reflects the relatively small sample size and the absence of pre-illness measures of muscle mass from which to estimate net loss. In future studies it would be important to characterise further the likely parallel between metabolic syndrome, the cancer patient with sarcopenic obesity and the mechanisms that underlie the present observed changes in skeletal muscle ultrastructure.

The controls in the current study were younger than the cancer patients. It has been suggested that ageing results in an increase in intramyocellular lipid content (Crane et al., 2010). Whilst this may be a contributing factor to the lower numbers of lipid droplets in the control group, the observed association between weight-loss and lipid droplets in the cancer cohort was independent of age. Likewise, there were no observed differences in lipid droplet numbers between men and women ($p=0.630$) or tumour site (one-way ANOVA, $p=0.559$; see Figure 8.2). However, given that the numbers in each group were small, the influence of these factors would be better explored in a larger dataset.

The likely mechanism for the increased deposition of lipid within the skeletal muscle of weight losing cancer patients probably reflects an imbalance between fatty acid supply and utilisation. The enhanced lipolysis in cancer cachexia has been long established. However, recent research has focused more closely on mitochondrial dysfunction and, as observed in sepsis (Fredriksson et al., 2008), impaired mitochondrial fatty acid oxidation/oxidative capacity may contribute to lipid accumulation. Of interest, mitochondrial derangement has been demonstrated in COPD muscle wasting (Gosker et al., 2007). The present study did not extend to a detailed evaluation of myocellular mitochondrial ultrastructure or function but clearly this could be the focus of further research.

In summary, this study suggests that the presence of lipid droplets is related to the presence of cancer and increases with weight-loss. The specific mechanisms/drivers of this phenomenon remain to be elucidated.

Chapter 9: **Impact of cachexia on longitudinal changes in muscle function and mass in upper gastrointestinal cancer patients undergoing surgery**

9.1 ***Abstract***

The original intention was to undertake a longitudinal natural history study of the development of cancer cachexia in patients following resection. It was anticipated that as patients developed recurrent disease cachexia would become more prevalent. As it happened, patients who returned for follow up were still disease free whereas those that developed recurrence (and presumably cachexia) died so quickly as not to be picked up during follow up. In order to make sense of the data that had been collected, this Chapter describes a longitudinal study (mirroring the approach taken in the biomarker discovery studies of Part I) carried out to assess the impact of pre-operative cachexia on short-term post-operative outcome.

At baseline, 42 UGI cancer patients were recruited: 10 had palliative surgery and 32 a "curative" resection. Palliative patients had poorer performance status and an increased sit-to-stand time compared with patients having "curative" resection. However, other anthropometric and functional measures did not differ significantly between the two groups. Only the patients in the "curative" group underwent repeated assessment at ~8 months post operatively (median 231 days). The palliative group did not survive sufficiently long to allow such follow up. In the "curative" group, 3 patients declined and 5 patients died before the second appointment.

Cachectic patients who underwent "curative" surgery had less post-operative weight-loss compared with non-cachectic patients (-0.7 vs. -4.5 % per 100 days respectively, p=0.034). By eight months post-operatively there were no significant alterations in quadriceps strength, lower limb power and HGD in either cachectic or non-cachectic patients. Cachectic patients demonstrated improved global QoL (p=0.039) following resection, whereas non-cachectic patients had significant deterioration in TSF thickness (p=0.049), MAMC (p<0.001), weight (p=0.001) and sit-to-stand (STS) time (p=0.024). In summary, this chapter showed that in patients

with UGI cancer undergoing potentially curative resection, those who were nutritionally replete pre-operatively had ground to lose post-operatively. In contrast, those who were cachectic pre-operatively benefitted from removal of the tumour and suffered attenuated loss from a low baseline. This study shows the difficulties of longitudinal data collection and how some of the baseline variables and clinical issues can impinge on the tracking of development or recovery from cachexia.

9.2 Introduction

Chapters 7 and 8 have established that muscle function, mass and quality are influenced by cachexia and gender, and that there is an increase in intramyocellular lipid droplets with cachexia that may impact on muscle quality. What is not clear is whether these changes at the time of surgery are reversible after tumour resection and also what impact progressive cachexia may have on subsequent recovery. As discussed previously, the initial protocol for the intended longitudinal study was not achieved. The original experimental model was to recruit patients at baseline, whilst undergoing surgery, and then follow them up every 6 months to identify a sub-population who developed recurrent cancer cachexia. This latter group would comprise those who had a 'curative' procedure but had developed recurrent tumour and weight-loss, and a group from those who had a 'palliative' procedure with tumour still present who continued to lose weight. However, as explained in Chapter 5 and as will be discussed further in Chapter 10, this was not possible. Thus, an alternative model was developed for the longitudinal follow up of muscle function and mass. Similar to the molecular studies, patients undergoing potentially curative resection of tumour, with or without cachexia, were followed up and functional measurements compared at these time points. Patients who were disease free at follow up were included in the analysis, and in order to try and achieve adequate numbers, the first follow up assessment (i.e. ~8 months) was used.

In 2007 there were 23000 new cases of UGI cancer (UK) and surgery remains the mainstay of treatment providing potential cure. However, major surgery is associated with semi-starvation, stress response, abnormal post-operative immune

function and increased protein catabolism with resultant sequelae (Fearon et al., 2013, Gustafsson and Ljungqvist, 2011). Furthermore, even in cancer patients who undergo curative surgery 5 year survival is poor – for example, ~20% following oesophageal resection (Hulscher et al., 2001) and ~10% following Whipples pancreaticoduodenectomy (Conlon et al., 1996). Although many cancer patients are weight losing at diagnosis, there are few studies examining the impact of cachexia on post-operative progress. In pancreatic cancer, cachectic patients (weight-loss >10%) were more likely to have unresectable tumours and have poorer survival than those who were not cachectic pre-operatively (Bachmann et al., 2008, Bachmann et al., 2009).

With this in mind, the first part of this chapter is a comparison of the baseline phenotype of patients undergoing curative resection versus palliative surgery. The second section investigates the impact of pre-operative cachexia on short-term post-operative recovery of muscle function/mass and QoL in patients who had a curative resection. For the reasons outlined previously it was not possible to use the present longitudinal data to examine the predictive value of the molecular biomarkers identified in Part I for the long-term development of progressive cachexia.

9.3 ***Hypotheses***

- Patients undergoing palliative surgery would have poorer muscle function and survival compared to patients undergoing potentially curative resectional surgery.
- Patients with pre-operative cachexia would have poorer muscle function, increased loss of muscle and a poorer QoL in the short-term following tumour resection.

9.4 ***Methods***

Participants: see Section 2.1

Anthropometric assessments: see Section 2.2

Blood collection: see Section 2.8.1

Blood measures (CRP, albumin): see Section 2.9

Muscle function and functional ability (IKES, LLEP, HGD, STS, TUG): see Section 2.3

MR imaging and analysis: see Section 2.6.1

Muscle mechanical quality: see Section 2.7

QoL (EORTC QLQ-C30, KPS): see Section 2.4

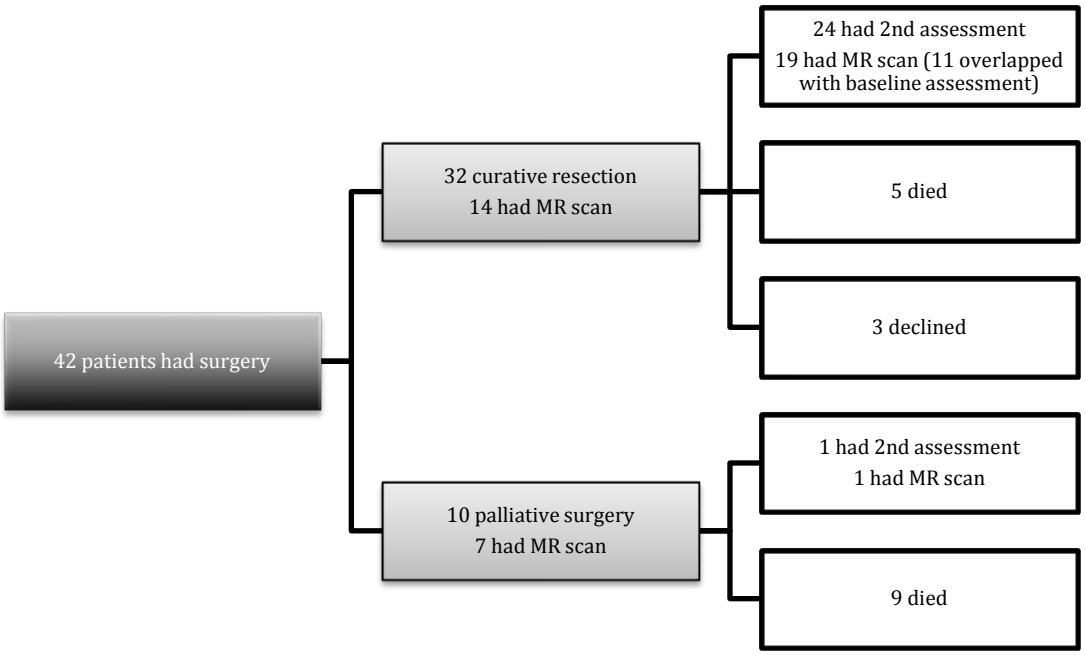
Statistics: see Section 2.12.1

9.5 ***Results***

UGI cancer patients identified to undergo potentially curative resection were included in the study. At the time of surgery, if patients had tumour that was unresectable, underwent a palliative procedure (e.g. bypass) or had evidence of metastases they were classified as 'palliative'. If patients underwent a successful tumour resection, they were classified as 'curative'. A weight-loss above 10% of pre-illness weight was used to define cachexia. Patients were followed up at approximately 8 months post-operatively. The results of the cross-sectional study in Chapter 7 suggested sexual dimorphism in the response of muscle to cachexia. However, due to the limited numbers of patients, further sub-dividing by gender prohibited meaningful analysis. For example there was only one female cachectic patient who had an MR scan. There were also some measures that were not performed in all patients either due to patient preference (e.g. blood sample), or lack of availability (e.g. limited time slots for MR imaging).

Of the 42 patients recruited in total, 32 had a 'curative' operation and 10 had a 'palliative' operation. Patients had assessments immediately pre-operatively, and repeated measures were carried out at a median of ~8 months (231 days; range 134-425 days) post-operatively. Those in the palliative group were only analysed at baseline (due to n=1 at follow up). Figure 9.1 illustrates the outcome at follow up and the subtotal who underwent MR imaging of the quadriceps muscles.

FIGURE 9.1– OUTCOME AT TIME OF FIRST FOLLOW UP (~8 MONTHS) FOR PATIENTS UNDERGOING SURGERY



Patients were classified as curative or palliative dependant upon whether the primary tumour was resected at time of surgery and whether there was evidence of metastases. An attempt to follow-up all patients was made. 16% of patients who underwent curative resection and 90% of patients who had palliative surgery had died before the 2nd assessment. Of the patients who underwent MR scans, only 11 in the curative group had a scan at both baseline and follow-up assessment. Abbreviations: MR, magnetic resonance.

Baseline phenotype of patients undergoing curative resection or palliative surgery

Demographics are illustrated in Table 9.1. In the curative cohort, 6 patients had gastric cancer, 13 oesophageal, 4 OGJ, and 9 had pancreatic cancer. The majority (n=29) of patients had adenocarcinoma and 3 patients had squamous cell carcinoma (oesophageal). 5 patients had stage 1 cancer, 7 stage 2, 19 stage 3 and 1 stage 4 cancer. 15 patients had undergone neoadjuvant chemotherapy and 17 had not.

In the palliative cohort, 2 patients had gastric cancer and 8 had pancreatic cancer. All 10 had adenocarcinoma. 5 patients had stage 3 and 5 had stage 4 cancer. 1 patient had undergone neoadjuvant chemotherapy and 9 had not.

Patients undergoing palliative surgery had significantly lower KPS and increased STS time compared with those undergoing curative resection. There was also a trend towards lower TSF in palliative compared with curative cohort. However, quadriceps CSA (derived from mid-femur MR scans) were similar and the proportion of patients who were cachectic did not significantly differ between the two groups. Patients who had a curative resection had significantly greater median survival compared with the palliative group (985 vs. 245 days respectively, Log-Rank $p<0.001$). Figure 9.2 illustrates the Kaplan-Meier survival curves for each group.

Table 9.1: Demographics, anthropometry, muscle function, functional ability and muscle mass for patients undergoing 'curative' vs. 'palliative' surgery

A. Total cohort

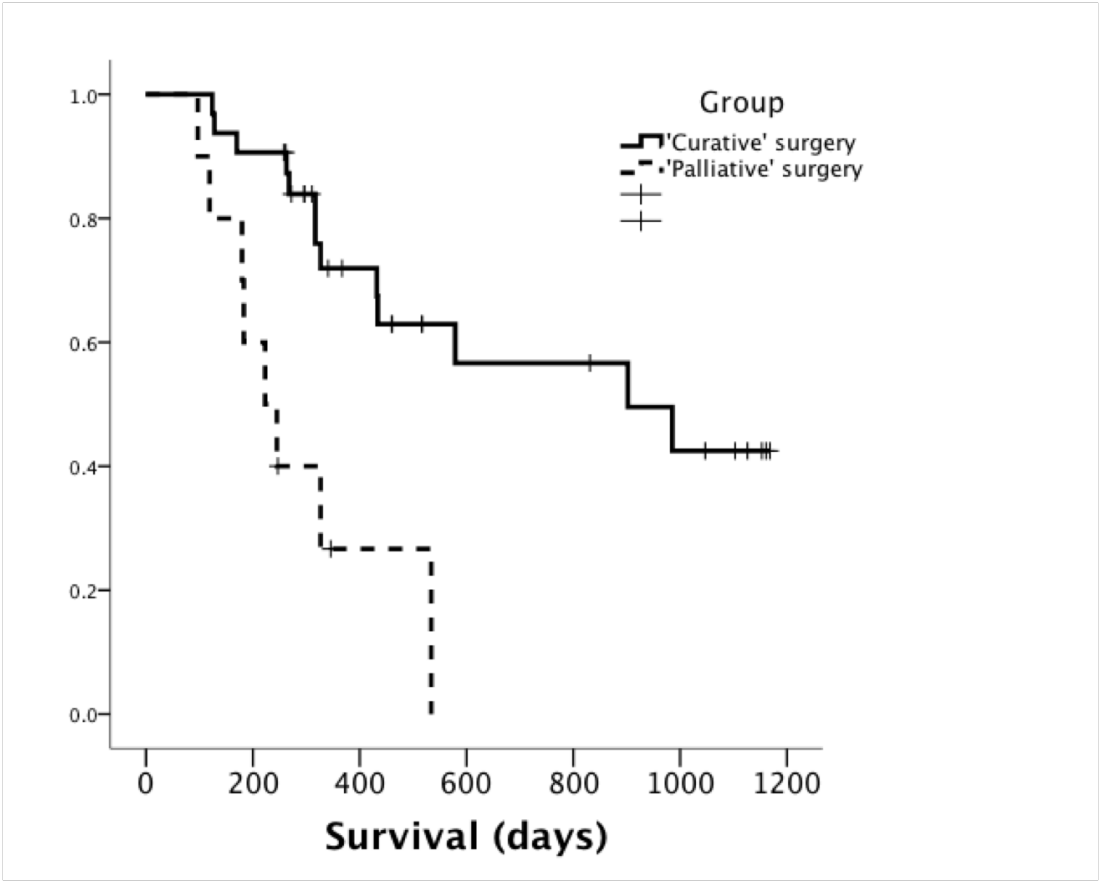
	'Curative' surgery (n=32)	**'Palliative' surgery** (n=10)	p value
Age (years)	65.1 (9.2)	62.9 (11.8)	0.541
M/F	21/11	6/4	0.746
KPS	85.6 (11.9)	75.0 (16.5)	**0.031**
Height (cm)	169.8 (8.4)	167.8 (10.5)	0.539
Weight-loss (%)	-6.6 (7.8)	-10.3 (13.5)	0.429
Cachexia Y/N	13/19	3/7	0.817
Weight (kg)	76.2 (16.0)	67.3 (13.8)	0.122
BMI (kg/m^2)	26.3 (4.7)	23.7 (3.3)	0.113
MAC (cm)	30.0 (3.5)*	27.2 (2.3)	**0.023**
TSF (mm)	16.4 (7.4)*	11.8 (5.1)	0.076
MAMC (cm)	24.8 (2.7)*	23.5 (2.3)	0.179
Arm CSA (cm^2)	40.7 (10.0)*	35.7 (7.2)	0.153
Strength (N/Kg)	3.6 (1.2)**	3.3 (1.1) **	0.527
Power (W/Kg)	1.3 (0.5)*	1.3 (0.3)*	0.999
HGD (kg)	35.3 (10.5)*	28.7 (9.4)	0.098
STS (s)	0.6 (0.2)***	0.8 (0.1)**	**0.010**
TUG (s)	6.8 (1.3)***	7.5 (1.4)**	0.195

B. MR imaging cohort

	'Curative' surgery (n=14)	**'Palliative' surgery** (n=7)	p value
Q. CSA (mm^2)	5278.7 (1268.6)	5332.1 (1524.1)	0.933
Q. CSA/height^2 (mm^2/m^2)	1902.1 (325.2)	1873.7 (322.4)	0.975
Fat free Q. CSA (mm^2)	4390.1 (1182.8)**	4585.5 (1454.4)	0.753
Fat free Q. CSA/height^2 (mm^2/m^2)	1604.3 (321.4)**	1606.4 (329.7)	0.989
Fat infiltration (%)	13.7 (6.0)**	14.7 (4.4)	0.718

*Results are presented as mean (SD) or categorically. Student's t-test or Fisher's exact test were used to compare values. Not all variables could be measured in all patients: *=n-1, **=n-2, ***=n-4. Abbreviations: M, male; F, female; KPS, Karnofsky performance score; BMI, body mass index; MAC, mid arm circumference; TSF, triceps skin fold; MAMC, mid arm muscle circumference; CSA, cross-sectional area; Q. CSA, mid-femur quadriceps cross-sectional area; HGD, hand grip dynamometry; STS, sit-to-stand; TUG, 3m timed up-and-go; MR, magnetic resonance; cm, centimetres; mm, millimetres; kg, kilograms; N, newtons; W, watts; s, seconds; m, metres.*

FIGURE 9.2: POST-OPERATIVE SURVIVAL CURVES FOR PATIENTS WHO HAD 'CURATIVE' COMPARED WITH 'PALLIATIVE' SURGERY



Kaplan-Meier survival analysis was done comparing 'curative' and 'palliative' surgery groups; median survival 985 vs. 245 days respectively, Log Rank p<0.001.

The impact of cachexia on post-operative recovery in patients undergoing curative surgery

Patients in the 'curative' group were further divided for analysis into those who were cachectic (weight-loss >10% - given that this weight-loss relates to surgical risk (Windsor and Hill, 1988) pre-operatively and those who were not cachectic (weight-loss <10%). Repeated measures at the first follow-up appointment were compared with baseline values.

The demographics of patients undergoing curative resection divided into cachectic and non-cachectic groups are illustrated in Table 9.2. Cachectic patients had significantly increased weight-loss compared with non-cachectic patients, but other demographic variables were similar. 75% of patients re-attended at a median of 231 days (~ 8 months) post-operatively and underwent repeat measures.

Blood measures

Results for albumin and CRP are illustrated in Table 9.3. Serum albumin concentration increased significantly at the follow up assessment compared with baseline in both cachectic (41.2 vs. 34.0 g/l, p=0.002) and non-cachectic patients (42.4 vs. 36.7 g/l, p=0.001). Mean CRP levels were higher in cachectic than non-cachectic patients at baseline, but this was not significant (36.7 vs. 5.3 mg/l, p=0.450). Likewise, follow up CRP levels did not significantly differ from baseline values in either group.

Table 9.2: Patient demographics for those undergoing 'curative' surgery according to the presence or absence of cachexia

		Cachexia (n=13)	No cachexia (n=19)	p-value
Age (years)		63 (7)	66 (10)	0.323
M/F		8:5	13:6	0.981
Tumour stage	1	2	3	0.748
	2	2	5	
	3	9	11	
	4	0	0	
Tumour site	Oesophagus	3	9	0.244
	Stomach	3	4	
	OGJ	1	3	
	Pancreas	6	3	
Height (cm)		170 (7)	168 (8)	0.541
Weight (kg)		71.8 (12.4)	78.1 (17.9)	0.276
BMI (kg/m^2)		24.8 (3.5)	27.5 (5.1)	0.107
Weight-loss (%)		14.5 (4.9)	1.7 (4.4)	**<0.001**
Time baseline to follow up (days)		217 (62)	235 (68)	0.522

Results are presented as mean (SD) or categorically. Comparison between groups was made using Student's t-test/Fisher's exact test. Abbreviations: M, male; F, female; OGJ, oesophago-gastric junction; BMI, body mass index; cm, centimetres; kg, kilograms; m, metres.

Anthropometry

Anthropometric variables (Table 9.3) were similar in cachectic patients at follow up compared with baseline assessments (weight 72.9 vs. 71.5 kg, p=0.465; BMI 24.8 vs. 24.2 kg/m^2, p=0.401; TSF 13.4 vs. 13.8 mm, p=0.699; MAMC 23.7 vs. 24.5 cm, p=0.123 respectively). In non-cachectic patients, all measures significantly fell at follow up assessment compared with baseline (weight 69.0 vs. 79.7 kg, p=0.001; BMI 24.0 vs. 27.6 kg/m^2, p=0.001; TSF 14.1 vs. 18.1 mm, p=0.049; MAMC 23.3 vs. 25.6 cm, p<0.001 respectively). In order to control for potential influence of variation in time from baseline to follow up, % change per 100 days was calculated for each measure (Figure 9.3). Using this, weight decreased at a significantly higher rate in non-cachectic than in cachectic patients (-4.5% vs. -0.7%, p=0.034). However, for the other variables, there were no significant differences between the groups in % change per 100 days.

Muscle mass

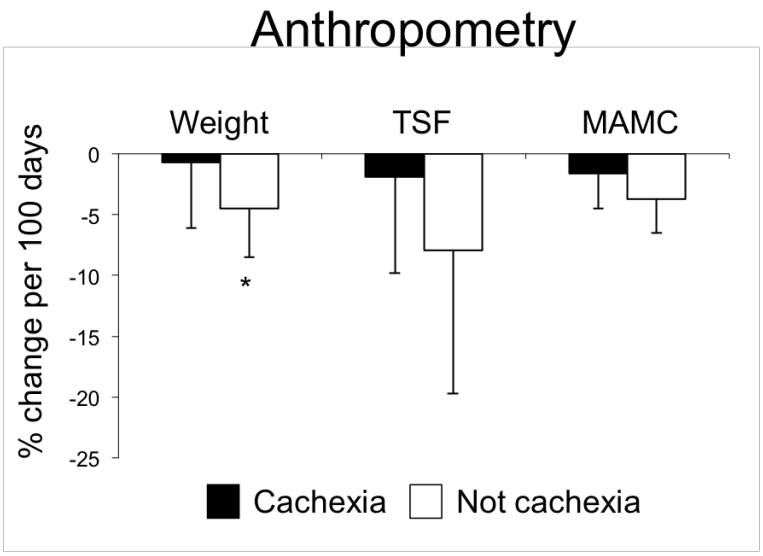
Mid-femur quadriceps CSA was measured using MR scan and mid-arm muscle CSA was calculated from anthropometric values using previously validated formulae (Heymsfield et al., 1982). Table 9.4 illustrates the results. In cachectic patients, quadriceps CSA and arm muscle CSA did not differ significantly between follow up and baseline (quads CSA 1823 vs. 1832 cm^2/m^2, p=0.904; arm CSA 36.7 vs. 39.5 cm^2, p=0.122 respectively). When expressed as change over time, mean quads CSA change was 0.4 %/100 days and mean arm muscle CSA change was -3.8 %/100days (Figure 9.4).

Table 9.3: Baseline and follow-up blood measures and anthropometry for UGI cancer patients (with and without cachexia) undergoing 'curative' surgery.

	Cachexia		**p-value**	**Not cachexia**		**p-value**
	Baseline	Follow-up		Baseline	Follow-up	
	(n=7)	(n=7)		(n=11)	(n=11)	
Albumin	34.0 (3.4)*	41.2 (2.9)*	0.002	36.7 (3.7)	42.4 (3.4)	0.001
CRP	36.7 (56.5)	13.0 (16.5)	0.346	5.3 (8.5)	9.2 (12.2)	0.450
	(n=9)	(n=9)		(n=15)	(n=15)	
Weight (kg)	71.5 (14.8)	72.9 (12.3)	0.465	79.7 (19.8)	69.0 (14.1)	0.001
BMI (kg/m^2)	24.2 (4.1)	24.8 (3.9)	0.401	27.6 (5.4)	24.0 (3.6)	0.001
TSF (mm)	13.8 (3.9)*	13.4 (5.0)*	0.699	18.1 (8.7)	14.1 (6.1)	0.049
MAMC (cm)	24.5 (2.6)*	23.7 (3.1)*	0.123	25.6 (3.0)	23.3 (2.7)	<0.001
* = n-1						

Results are presented as mean (SD). Comparison between baseline and follow-up measurements was made using paired t-test. Abbreviations: UGI, upper gastrointestinal; CRP, C-reactive protein; BMI, body mass index; TSF, triceps skin fold thickness; MAMC, mid-arm muscle circumference; kg, kilogram; m, metres; mm, millimetres; cm, centimetres.

FIGURE 9.3: PERCENTAGE CHANGE IN ANTHROPOMETRY PER 100 DAYS IN UGI CANCER PATIENTS (WITH AND WITHOUT CACHEXIA).



*The % change per 100 days between baseline and follow-up assessments was calculated. Comparison of cachectic with not cachectic patients was done using Student's t-test. * p<0.05 compared with cachectic. Abbreviations: UGI, upper gastrointestinal; TSF, triceps skinfold thickness; MAMC, mid-arm muscle circumference.*

In non-cachectic patients, follow up compared with baseline assessments demonstrated a trend towards a fall in quads CSA (1836 vs. 2081 cm^2/m^2, p=0.063) and a significant fall in arm muscle CSA (34.9 vs. 43.6 cm^2, p<0.001). Change over time in non-cachectic patients was -4.3 %/100 days for quads CSA and -8.2 %/100 days for arm CSA. These values were not significantly different from the cachectic cohort (p=0.120 and p=0.123 respectively, Figure 9.4).

Muscle function and functional ability

Quadriceps strength, lower limb power and HGD did not differ between baseline and follow up for either cachectic or non-cachectic patients (Table 9.4). When values were expressed as change over time (Figure 9.4), although lower limb muscle function increased in cachectic more than non-cachectic patients, this was not significant (strength, 17.5 vs. 2.6 %/100 days respectively, p=0.127; power, 8.6 vs. 3.9 %/100 days respectively, p=0.436). Only HGD significantly improved in cachectic compared with non-cachectic patients (4.0% vs. -1.9%, p=0.043). STS time and 3m timed up-and-go (TUG) were similar in cachectic patients at baseline and follow up (Table 9.4). In non-cachectic patients, there was a significant rise in STS time at follow up compared with baseline (0.63 vs. 0.55 s, p=0.024) but not with TUG measures.

Muscle quality

Muscle quality (quadriceps strength/unit CSA) was not significantly different between cachectic and non-cachectic groups or time points (Table 9.4). The % change in muscle quality per 100 days was higher in cachectic compared with

Table 9.4: Baseline and follow-up muscle mass, function, functional ability and quality for UGI cancer patients (with and without cachexia) undergoing 'curative' surgery.

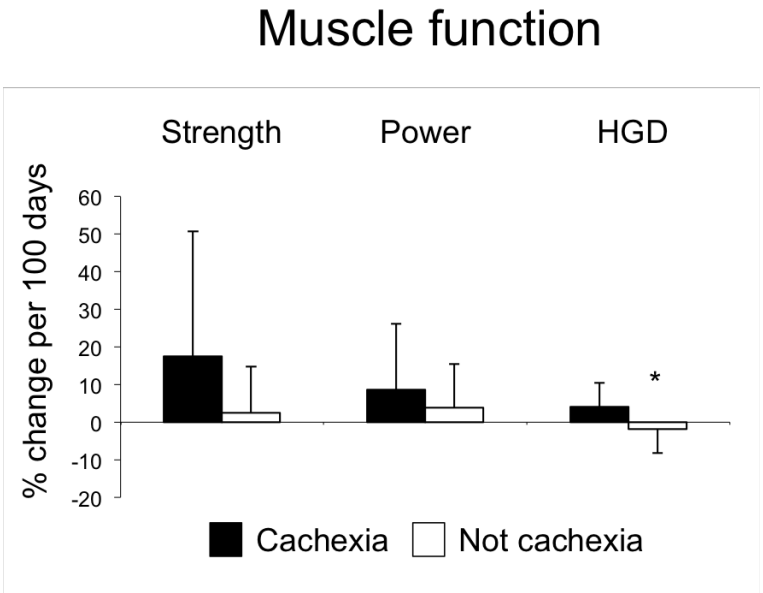
	Cachexia		**p-value**	**Not cachexia**		**p-value**
	Baseline	Follow-up		Baseline	Follow-up	
	(n=8)	(n=8)		(n=15)	(n=15)	
A. CSA	39.5 (9.0)	36.7 (10.2)	0.122	43.6 (11.5)	34.9 (9.3)	<0.001
	(n=6)	(n=6)		(n=5)	(n=5)	
Q. CSA	1832 (169)	1823 (301)	0.904	2061 (496)	1836 (367)	0.063
	(n=9)	(n=9)		(n=15)	(n=15)	
HGD	35.1 (7.8)	37.0 (8.3)	0.173	37.7 (12.9)*	35.2 (10.3)*	0.103
Quads strength	3.1 (1.0)	3.7 (0.6)	0.118	3.8 (1.4)	3.9 (1.2)	0.924
Lower limb power	1.2 (0.5)	1.4 (0.4)	0.308	1.4 (0.5)	1.5 (0.6)	0.379
	(n=8)	(n=8)		(n=12)	(n=12)	
STS	0.67 (0.25)	0.65 (0.18)	0.628	0.55 (0.16)	0.63 (0.13)	0.024
TUG	6.9 (1.1)	6.6 (1.0)	0.455	6.6 (1.2)	6.9 (1.6)	0.179
	(n=6)	(n=6)		(n=5)	(n=5)	
Muscle quality	0.04 (0.02)	0.05 (0.01)	0.384	0.05 (0.01)	0.05 (0.01)	0.486

* = n-1

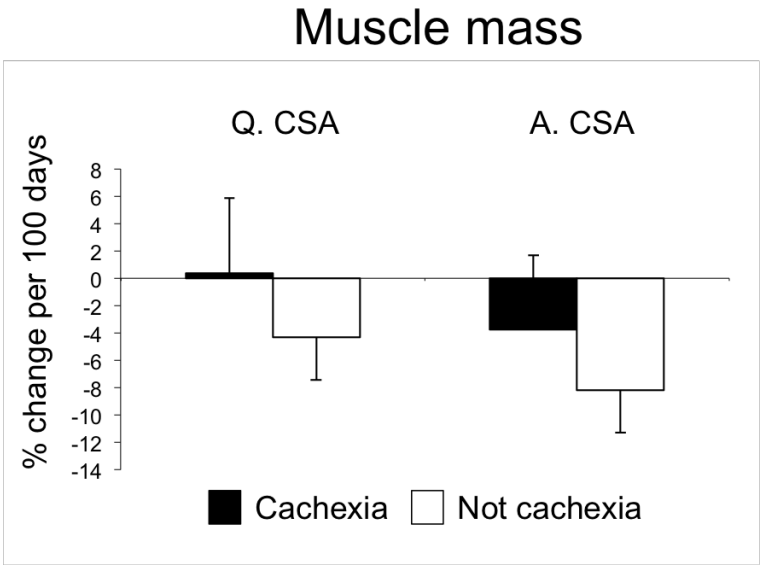
Results are presented as mean (SD). Comparison between baseline and follow-up measurements was made using paired t-test. Abbreviations: UGI, upper gastrointestinal; STS, sit-to-stand time; TUG, 3m timed up-and-go; Q. CSA, mid-femur quadriceps muscle cross-sectional area; A. CSA, mid-arm muscle area; HGD, handgrip dynamometry.

FIGURE 9.4: PERCENTAGE CHANGE IN MUSCLE MASS, FUNCTION AND QUALITY PER 100 DAYS IN UGI CANCER PATIENTS (WITH AND WITHOUT CACHEXIA).

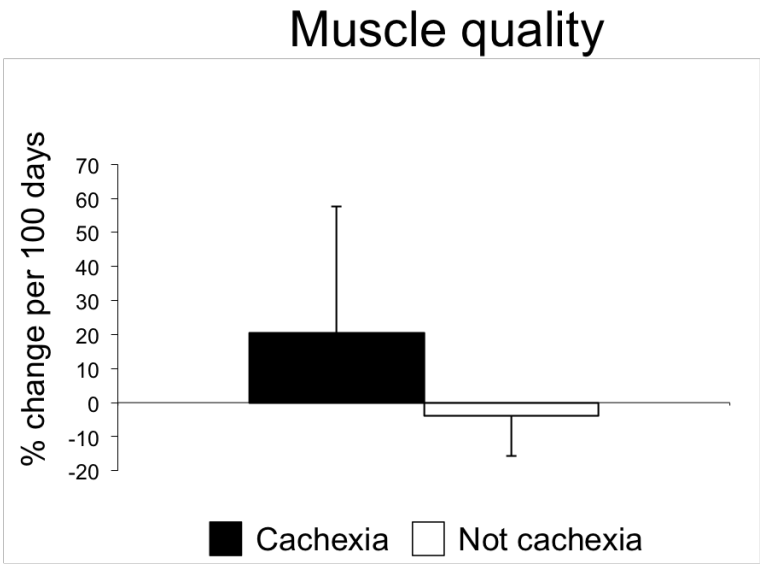
A.



B.



C.



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*The % change per 100 days between baseline and follow-up assessments was calculated for muscle function (**A.**), muscle mass (**B.**), and muscle quality (**C.**). Comparison of cachectic with not cachectic patients was done using Student's t-test. * p<0.05 compared with cachectic. Abbreviations: UGI, upper gastrointestinal; Q. CSA, mid-femur quadriceps muscle cross-sectional area; A. CSA, mid-arm muscle cross-sectional area; HGD, handgrip dynamometry.*

non-cachectic patients, but the difference was not significant (20.4 vs. -3.8 %/100 days, p=0.200 respectively, Figure 9.4).

QoL

The EORTC-QLQ C-30 was used to assess QoL (Table 9.5). In cachectic compared with non-cachectic patients, baseline global QoL was significantly lower (86.1 vs. 143.6, p=0.011) and there was a trend toward the fatigue score being higher (44.5 vs. 29.9, p=0.078). In cachectic patients, global QoL significantly improved at the follow up assessment (138.9 vs. 86.1, p=0.039). KPS and physical function scores were similar between baseline and follow up in both cachectic and non-cachectic patients.

Table 9.5: Baseline and follow-up QoL for UGI cancer patients (with and without cachexia) undergoing 'curative' surgery.

	Cachexia		**p-value**	**Not cachexia**		**p-value**
	Baseline (n=6)	Follow-up (n=6)		Baseline (n=13)	Follow-up (n=13)	
Global QoL	86.1 (71.0)	138.9 (39.0)	0.039	143.6 (40.6)	141.0 (37.6)	0.790
Fatigue	44.5 (42.8)	29.6 (26.0)	0.158	29.9 (26.2)	32.5 (19.5)	0.487
Physical function	77.8 (30.6)	82.2 (27.2)	0.602	84.5 (23.7)	80.0 (15.6)	0.187
KPS	87.1 (12.5)*	88.6 (9.0)*	0.736	85.7 (10.9)*	85.0 (10.2)*	0.807

* = n+1

Results are presented as mean (SD). Comparison between baseline and follow-up measurements was made using paired t-test.
Abbreviations: QoL, quality of life; KPS, Karnofsky performance score; UGI, upper gastrointestinal.

9.6 Discussion

Comparison of 'palliative' versus 'curative' patients at baseline

The patients in the 'palliative' group had a higher proportion of advanced disease (i.e. 50% stage 4 cancer vs. 3% in curative group). Likewise, the median survival for palliative vs curative cohorts was significantly reduced. Due to this, it was not possible to carry out any follow up analysis of the palliative cohort as 90% of this cohort had died before a follow up assessment could be carried out. Even in those patients who had undergone 'curative' resection, 16% had died prior to follow up. These figures emphasise the devastating impact that UGI cancer can have on patients and highlights that even in those who are deemed suitable for potentially curative surgery, outcome is still very guarded. The results are in keeping with other studies where, for example, ~10-22% of patients with oesophageal cancer undergoing surgery failed to return to a 6 month follow up appointment (Blazeby et al., 2005a, Barbour et al., 2008, Djarv et al., 2008). Reasons for the poor post-operative survival in this group will include progression of disease, co-morbidities and complications of major surgery, and it is also likely that a reasonable proportion of these patients would deteriorate with progressive cachexia. The poor outcome in the palliative group suggests a pressing need for research into methods that allow better pre-operative staging and patient selection in order to avoid exposing patients to the potential risks of major surgery when symptomatic treatment may be more appropriate. Previously, it has been reported that pre-operative cachexia (weight-loss >10%) reduced the likelihood of a curative resection in patients with pancreatic cancer and was associated with more advanced disease and poorer survival (Bachmann et al., 2008, Bachmann et al., 2009). The results of the current study

contrast with this as only 30% of the palliative group were cachectic pre-operatively compared with 41% in the curative surgery group. This may reflect the inclusion of oesophageal and gastric in addition to pancreatic cancer patients in the current study, or perhaps some aspect of tumour biology which resulted in a more aggressive disease in the palliative cohort. Alternatively, the palliative patients may have been late on in the cachexia journey and had some degree of progressive fluid retention which contributed to their apparent lack of weight-loss. It is interesting that the only significant differences between the curative and palliative groups were a reduced performance score and an increased STS time. This latter test is a measure of functional ability and the fact that this deteriorated in preference to any of the other functional measures suggests that it may be useful as a marker of early frailty. However, the numbers in the study are small introducing the potential for a Type II error.

Longitudinal phenotypic changes in the presence or absence of cachexia for patients undergoing potentially curative resection

The fact that, in the curative group, both cachectic and non-cachectic patients had significantly increased albumin levels following surgery suggests that by ~8 months there has been recovery from the negative nutritional consequences of cancer. With albumin being a negative acute phase protein, improved levels of albumin may also reflect abrogation of an acute phase response to the presence of a tumour. However, although the mean CRP levels were higher in cachectic patients at baseline, there

were no significant differences following surgery, nor were levels significantly different from non-cachectic patients.

There are relatively few longitudinal studies assessing muscle function in human cancer cachexia, and those that are published tend to use QoL derived physical function scores. In the current study, patients who were disease free at follow up and had undergone a curative resection demonstrated differences in recovery post operatively according to the degree of cachexia at the time of surgery (Table 9.4).

Contrary to the initial hypothesis, cachectic patients did not fare worse in the short term following curative resectional surgery. Indeed, they appeared to maintain many of the muscle functional, mass and other variables. Furthermore, QoL improved at follow up compared with baseline. In contrast, there appeared to be some deterioration post-operatively for some of the measured variables in the non-cachectic patient group (i.e. weight, TSF, MAMC, quads and arm muscle CSA). The reasons for this intriguing observation are not clear. One might anticipate that patients who are weight losing pre-operatively would be frailer and would experience increased morbidity and mortality in the post-operative period. Indeed, previous studies in pancreatic cancer patients undergoing surgery have shown poorer outcomes in cachectic (weight-loss >10%) individuals (Bachmann et al., 2008, Bachmann et al., 2009, Pausch et al., 2012). In contrast, other authors have failed to demonstrate a clear relationship between pre-operative body composition and subsequent survival in patients undergoing pancreatic resection (Aslani et al., 2010).

Table 9.6 Summary of differences in longitudinal phenotypic changes in cancer patients (with and without cachexia) undergoing 'curative' surgery.

	Cachexia	No cachexia
Weight	⇔	⇓
TSF thickness	⇔	⇓
MAMC	⇔	⇓
Quadriceps strength	⇔	⇔
Lower limb power	⇔	⇔
HGD	⇔/⇑	⇔
Global QoL	⇑	⇔
Physical function score	⇔	⇔
Fatigue score	⇔	⇔
KPS	⇔	⇔
Quadriceps CSA	⇔	⇓
Arm muscle CSA	⇔	⇓

Overall post-operative change in anthropometry, muscle function, mass and QoL in patients undergoing 'curative' resectional surgery. Time period between measurements was ~8 months. ⇔ = no change in variable, ⇓ = decline in variable, ⇑ = improvement in variable. This summary does not inform on what might have happened between assessments (i.e. if there was initial decline followed by improvement resulting in overall no change in variable). Abbreviations: TSF, triceps skin fold; MAMC, mid arm muscle circumference; HGD, hand grip dynamometry; QoL, quality of life; KPS, Karnofsky performance score; CSA, cross-sectional area.

In this latter study, whilst patients had a reduction in total body fat pre-operatively, protein stores were preserved with only ~14% of patients being protein deficient at the time of surgery. This might suggest that the relative contribution of each tissue compartment to overall weight-loss may relate more strongly to post-operative progress and survival rather than gross weight-loss per se. The results of CT based body composition analyses demonstrating an adverse prognosis in patients with sarcopenic obesity in cancer cachexia would be supportive of this concept (Tan et al., 2009). Clearly, in the design of future cancer cachexia studies, it would be informative to utilise body composition measures to stratify patients at the outset rather than using them solely as outcome measures.

Although non-cachectic patients showed evidence of decline in anthropometric and muscle CSA measures, in the cachectic cohort all anthropometric and functional variables were relatively well preserved post-operatively. Given that all patients underwent similar major surgical resections, it would seem unlikely that there was no response to this surgical insult in the cachectic cohort. There are several studies examining the impact of major surgery on cancer patients although very few have been stratified by presence of cachexia. In patients undergoing open colorectal surgery, at 1 month post-operatively, weight, lean body mass, fat mass and physical performance (HGD/knee extension strength) had all reduced and there had been a rise in fatigue levels (Jensen et al., 2011). Likewise in patients undergoing oesophagectomy, physical function (6 minute walk and knee extensor strength) declined (Tatematsu et al., 2013) post-operatively. In gastric 'curative' surgery patients undergoing assessments pre-operatively and at 6 and 12 months, weight was

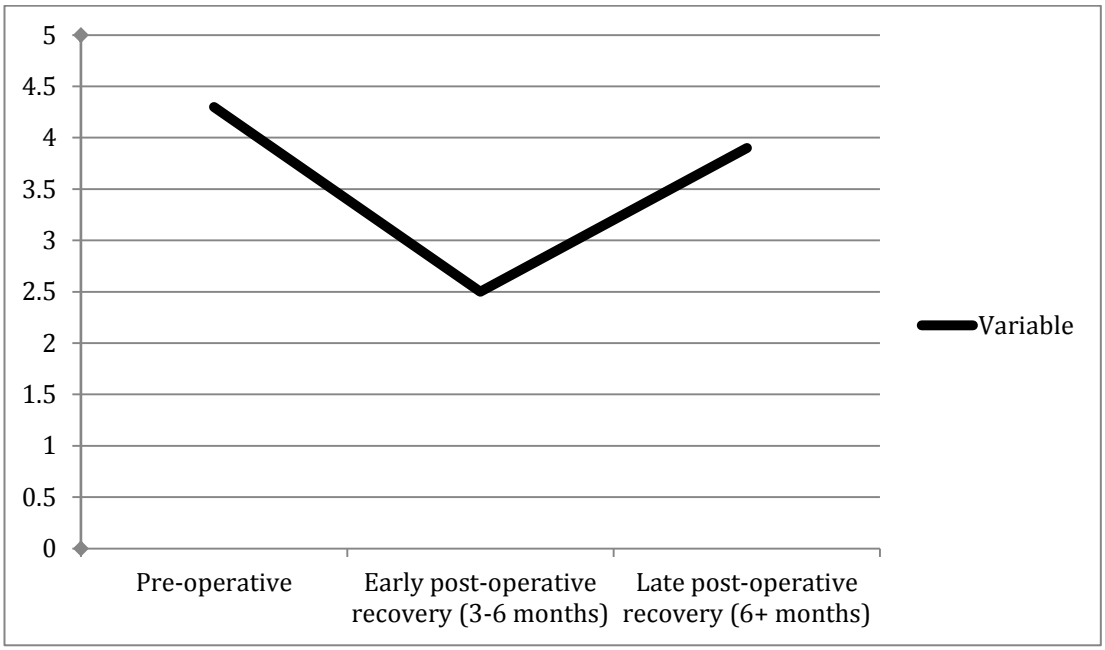
lost during the first 6 months post-operatively before stabilising primarily due to a decline in body fat of up to 40%. Body cell mass was unchanged post op and furthermore, there was no obvious deterioration in HGD (Liedman et al., 1997). Assessment of pancreatic cancer patients who underwent pancreatic resection (Whipples) at 4.6 years post surgery showed similar functional performance (respiratory muscle strength and hand grip strength) to patients undergoing cholecystectomy other than a lower body weight (Royall et al., 1996) although values were still above patients' 'ideal' weight. Conversely, in oesophageal cancer patients undergoing resection, those with the lowest hand grip strength compared to those with higher values had increased complications, hospital stay and mortality even compared with other risk factors (anaemia, hypoalbuminaemia, renal impairment, diabetes, increased MCV>100 and weight-loss) (Chen et al., 2011). These differences in functional outcome may reflect variation in follow-up time or assessment of different muscle groups (i.e. hand grip vs. quadriceps strength). The finding in Chapter 7 that HGD at baseline does not inform on load-bearing muscle function would reflect a similar concept that upper and lower limbs do not respond to the same degree to the insults of cachexia or major surgery.

In the current study, although a one-off follow up assessment at around 8 months allowed insight into post-operative changes, it does not inform on where patients may be on their recovery trajectory. There is general acceptance that the acute post-operative phase will have subsided within 6 months (Blazeby et al., 2005b, Blazeby et al., 2000, Zieren et al., 1996, Brooks et al., 2002, Viklund et al., 2006, Reynolds et al., 2006) after which a stabilisation and recovery phase occurs. However, this latter

phase can be variable and is dependent on what outcome is being measured. Furthermore, different research groups have reported contrasting results making it difficult to grasp the exact nutritional and physiological consequences of major surgery. For example, stabilisation of weight-loss and nutritional decline following pancreatic, oesophageal or gastric surgery after 4-6 months has been reported (Niedergethmann et al., 2006, Liedman et al., 1997, Ludwig et al., 2001, Melvin et al., 1998, McLeod et al., 1995) whereas other groups demonstrate persistent deterioration in these variables after the same operations (Martin and Lagergren, 2009, Ong et al., 2000, Carey et al., 2011). Some of these contrasting results may be explained by variation in follow up period, patient heterogeneity and perhaps differences in standards of post-operative care, but it highlights the need for further investigations. In the current study, it would seem likely that, rather than there being no impact of surgery in cachectic patients, the results reflect the timing of the follow up assessment whilst patients are in a recovery phase (Figure 9.5).

However, this would not account for significant deterioration in anthropometry and quads/arm muscle CSA seen in the non-cachectic patients over a similar follow up period post-operatively. One hypothesis is that the tissue loss experienced by cachectic patients pre-operatively resulted in a blunted response to the stress of surgery. That is, changes in anabolic and catabolic pathways brought about by host-tumour interaction had produced such a profound effect that the benefit of removing the pro-cachectic stimulus of the tumour outweighed the negative impact of major surgery, giving the appearance of greater recovery.

FIGURE 9.5: SCHEMATIC ILLUSTRATING POST-OPERATIVE RECOVERY OF, FOR EXAMPLE, WEIGHT, MUSCLE FUNCTION AND MASS



Assessment of patients at two time points (in the current Chapter, at pre-operative and late post-operative recovery stages) will not inform on what occurs in the interim.

The non-cachectic group did not have this same pre-operative change and thus they suffer the sequelae of major resection, although will still benefit from the potential for cure.

Muscle loss probably does not occur in a linear fashion or at a constant rate. It is conceivable that if muscle has already been lost, it becomes more difficult to continue tissue loss at the same rate. This concept may explain the deterioration in variables in non-cachectic patients where undergoing major surgery is the first stimulus to muscle loss whereas cachectic patients have already lost muscle and thus may have an abrogated response.

There will be many behavioural and social differences between patients, which will presumably have bearing on how they recover after surgery. For example, in the post-operative period, some patients may sit at home and eat only when they feel like it whereas others will be motivated to exercise and pay attention to their nutritional intake regardless of their symptomatology. These confounders were not addressed in the current study but an attempt to do so would require larger numbers of patients and would be extremely difficult due to the wide heterogeneity in behaviour amongst individuals.

Global QoL was poorer and fatigue levels higher in cachectic patients compared with non-cachectic patients at baseline. These differences diminished after removal of the tumour with a significant improvement in global QoL in cachectic patients. Changes in QoL after tumour resection have previously been reported. A positive impact on

QoL in patients undergoing surgery for pancreatic cancer has been shown (Kostro and Sledzinski, 2008). A more detailed look at QoL in patients undergoing pancreaticoduodenectomy at 1, 3, 6 and 12 months post surgery demonstrated an initial decline in QoL at 1 month compared with baseline before it improved significantly at 6 and 12 months (Chan et al., 2012). Patients undergoing oesophageal or gastric resection show a similar pattern of deterioration in QoL before values started to recover (Barbour et al., 2008, Viklund et al., 2006, Reynolds et al., 2006) with a further negative impact in patients undergoing neoadjuvant chemotherapy (van Meerten et al., 2008). Assessment of patients undergoing gastrectomy showed a fall in most QoL variables at 3 months which had improved at 12 month follow up. However, not all measures returned to normal with poorer fatigue, digestive symptoms, eating restrictions and body image disturbance persisting at 12 months (Kim et al., 2012). Indeed, in patients who survive fewer than 2 years after oesophageal surgery, QoL scores never return to baseline reported figures (Blazeby et al., 2000). Baseline QoL score has also been shown to relate to prognosis (Blazeby et al., 2005a).

Whereas some other investigators have reported persistent long-term deterioration in physical function scores after resectional surgery (van Meerten et al., 2008, Djarv et al., 2008, Lagergren et al., 2007), the current study did not demonstrate any differences in either cachectic or non-cachectic individuals. This is consistent with the lack of difference in most of the direct measures of muscle function. The reason for these differences compared with other studies is not clear, but may represent heterogeneity between patient groups, or some bias in patient selection. In non-

cachectic patients, despite a fall in weight and muscle CSA, QoL measures were similar pre and post surgery. This suggests that in these patients, any negative impact of surgery on QoL has diminished by this time, but also emphasises that the presence of cachexia is a main determinant of a poorer QoL.

Whilst the current study has yielded some interesting findings, the numbers are relatively small which may have given rise to a Type II error and hampered interpretation of some results. Chapter 7 highlighted a relationship between gender and the impact of cachexia on muscle function, mass and quality. It is possible that the response to surgery in the presence of cachexia is also influenced by gender, but this subdivision was not possible due to the small numbers limiting meaningful analysis. The type of tumour and/or operation may also be important. Previous studies have demonstrated post-operative differences in body composition and nutritional measures between patients undergoing laparoscopic, distal or total gastrectomy (Kiyama et al., 2005, Abdiev et al., 2011). Similarly, a comparison of patients with gastric, oesophageal or pancreatic cancer undergoing resection showed reduced weight-loss in those undergoing pancreatic surgery compared to the other two groups (Carey et al., 2011). Variability in QoL measures has also been reported according to surgery type (Barbour et al., 2008). Clearly, future longitudinal cachexia studies would be better served with larger numbers of both men and women to allow exploration of potential gender differences and variation according to operative technique.

In conclusion, this study demonstrates the difficulties of longitudinal follow-up studies in patients with advanced disease undergoing palliative surgery. In the present analysis only 10% of palliative patients were available for follow up at 6-8 months. In an ideal world, this would be the group that would have likely developed progressive cachexia and could have served to validate both molecular and physiological skeletal muscle biomarkers of cachexia. With regard to patients undergoing potentially curative surgery, the timescale of the follow-up in the present study was too short to allow for recurrence of disease and development of cachexia. In fact, those patients who were cachectic pre-operatively, tended to have stabilised from their cachexia as a result of tumour resection. This chapter also emphasises the negative impact cachexia can have on the QoL in patients with UGI cancer prior to undergoing surgery.

Summary of findings in Part II

The studies in Part II outline physiological assessment of skeletal muscle and exploration of the phenotype of UGI cancer patients with varying degrees of weight-loss undergoing surgery. Chapter 6 described the use of a novel k-means clustering technique to allow separation of contractile muscle tissue and non-contractile fatty tissue in MR scans from cancer patients. Female cancer patients appeared to have the smallest and highest fat content muscles compared with young and old healthy women. This finding emphasises the need to account for inter/intramuscular changes when assessing cross sectional imaging of muscle in cachectic patients, especially if comparing with a control group. Chapter 7 combined MR scans with muscle functional measures and QoL to assess the influence of cachexia on muscle mass, function and mechanical quality. It was demonstrated that there is variability in the impact of cachexia on muscle function, mass and quality according to gender and weight-loss. Furthermore, there appeared to be differences between upper and lower limb muscle compartments suggesting that certain muscle groups may be more susceptible to wasting than others. Due to the observation that muscles have increased fatty infiltration in cancer patients (Chapter 6) and muscle quality is reduced (Chapter 7), the study in Chapter 8 used TEM of skeletal muscle biopsies to investigate the relationship between cachexia and intramyocellular lipid droplets. Increased numbers of lipid droplets were seen in cancer patients compared with controls. Furthermore, the number of lipid droplets rose with progressive weight-loss. However, there was a negative correlation between lipid droplet number and CT derived measures of intermuscular fat suggesting that fat deposition inside and outside of myofibrils needs to be evaluated separately. The mechanisms responsible

for intramyocellular fat accumulation in cachexia remain to be elucidated. Finally, Chapter 9 dealt with a longitudinal study assessing the impact of pre-operative cachexia on post-operative functional recovery after major cancer resection. The difficulties of longitudinal follow-up studies in UGI cancer patients were highlighted. Around 16% of patients undergoing 'curative' surgery and 90% of those undergoing palliative surgery died before follow-up at 6-8 months. This study also emphasised that the presence of cancer cachexia is a key determinant of a poorer QoL. However, a surprising discovery was that pre-operative cachexia appeared to afford a degree of protection from deterioration in anthropometric and muscle CSA measures compared with non-cachectic patients following tumour resection. The reasons for this are not clear but may relate to a relatively greater benefit in removal of the pro-cachectic stimulus from the tumour compared with the impact of major surgery.

Chapter 10: **General discussion**

10.1 ***Overview of results and relevance to cancer cachexia***

For many years there has perhaps been an assumption that the mechanisms responsible for cancer cachexia, in general and for skeletal muscle in particular, in pre-clinical models are the same mechanisms responsible for muscle wasting in humans. This has been due to the strong evidence from many well conducted animal and cell studies. However, there has been a paucity of clinical data providing evidence to back up these assumptions. Variation in definitions of cachexia, the heterogeneity of patient populations and conflicting results between different human studies have made it difficult to distil what the true picture is. Furthermore, studies assessing the impact of cachexia on molecular or physiological aspects of skeletal muscle in humans have been lacking. The current thesis has contributed several key observations relating to the molecular pathways relevant and the physiological phenotype of skeletal muscle in cancer patients with cachexia.

In the first part of the thesis, the hypothesis-testing candidate skeletal muscle biomarker study (Chapter 3) demonstrated a lack of parallel with candidate markers, such as the atrogenes, relevant in preclinical models. This was further confirmed in the transcriptomic cross-sectional (Chapter 4) and longitudinal (Chapter 5) biomarker discovery studies where the atrogenes again failed to appear as prominent markers. Instead, the process of human cancer cachexia appears to relate more to relative hypo-anabolism with likely suppression of protein turnover. The results are particularly important in considering potential therapies for patients with cancer

cachexia. In the past, emphasis has perhaps been placed on targeting proteolytic pathways such as the UPP. However, the fact that this thesis suggests components of the UPP may not play the same role in human cachexia as perhaps assumed, may explain the lack of promising trial results. Perhaps drugs/therapies that are focused on overcoming the suppression in protein turnover and improving protein synthesis in the future may prove more successful. Other novel discoveries were changes in structural elements of the muscle (β-dystroglycan) or in compensatory pathways (CAMK2β) might be better potential biomarkers of cachexia in muscle. Clearly, these findings need confirmation before being proposed for validation studies.

The second part of the thesis centred on physiological assessment and phenotyping of skeletal muscle in patients with cancer cachexia. Although deterioration in muscle mass and function are thought to be key features of cancer cachexia, there are very few human studies directly assessing these variables. However, the wider availability of cross-sectional imaging has revolutionised body composition studies. The use of MR imaging has gained increasing favour due to its image detail and the low side-effect profile. The potential of a novel k-means technique to allow differentiation of muscle tissue and non-contractile 'fat' tissue in MR scans was assessed (Chapter 6). Female cancer patients were shown to have increased fatty infiltration of quadriceps muscle compared with young and elderly healthy female controls. This sets precedence for use of such imaging and analysis in future cachexia studies. Given such changes in muscle composition, a key question is whether cachexia is not only associated with muscle loss, but whether there is also a loss of muscle mechanical quality. Chapter 7 demonstrated that in men with cancer

and women with cachexia there was reduced muscle quality compared with controls. Another key finding was the observation that there may be gender differences in the impact of cachexia on muscle function, mass and quality in cancer patients. Although some previous studies have reported results suggesting differences between genders, this has never been explicitly explored and the majority of studies have analysed male and female body composition studies together. This may have contributed to misleading results if the distribution of genders differed between groups. Likewise, the suggestion that different muscle compartments respond differently to the same pro-cachectic stimuli is novel. Both strength (HGD vs. quads strength) and mass (anthropometry upper vs. MR imaging quadriceps) showed differences in impact of cachexia and also in recovery from major surgery. These factors need to be considered when deciding on the best endpoints for trials and also deserve further exploration.

Further exploration of altered muscle composition/quality in cachexia led to the novel observation that there are increased numbers of intramyocellular lipid droplets in skeletal muscle of cancer patients associated with increasing weight-loss (Chapter 8). The mechanisms for this remain to be investigated, but support other recently published data of cross-talk between adipose and muscle tissue in the cachectic patient. The findings of the longitudinal functional study (Chapter 9) highlighted the negative impact of cachexia on QoL pre-operatively and also that curative resection of the tumour burden allowed recovery of function/mass/QoL. One of the unexpected hurdles encountered in this thesis was the difficulty in attempting to carry out a longitudinal study in patients with cancer cachexia undergoing surgery

(Chapters 5 and 9). In particular, only 10% of patients who underwent 'palliative' surgery and 75% of those who had 'curative' surgery were available for follow-up at 6-8 months. The next section includes discussion about some of the reasons and potential solutions for this, but highlights that researchers need to be thoughtful in design of future studies.

In summary, this thesis has identified several potential skeletal muscle molecular biomarkers of cancer cachexia. However, the markers discovered are not consistent with pre-clinical models and therefore require further study before moving on to a validation programme. Physiological assessment of patients with cancer cachexia established the negative impact that cachexia can have on muscle mass, function, muscle quality and QoL, but demonstrated that the degree of impairment varies with sex and between muscle groups. Furthermore, the challenge of carrying out longitudinal studies in this patient group where frailty and clinical deterioration limit repeated assessments was highlighted. Such issues emphasise the need for a dual approach to the classification of cancer cachexia: if molecular markers prove difficult to discover or validate, then more specific and robust physiological indices of skeletal muscle mass and function may be the more important route to improve clinical trial design and cachexia classification.

10.2 Limitations and challenges

1. Definition

Limitation of weight-loss cut-off

It was highlighted in the main introduction to this thesis (Section 1.2.1) that for many years cancer cachexia has been poorly defined. This has been due in part to a lack of understanding of the molecular processes and the phenotype of human cancer cachexia. In this regard, the current thesis has attempted to investigate and contribute novel insights into these areas. There have also been several proposals for cachexia definitions published in recent years, and validation studies of these are currently being undertaken. For this thesis, a weight-loss of more than 5% (early biomarker studies) or 10% (phenotype studies) from pre-illness stable weight was used to define cachexia. However, using a simple cut-off may have confounded some of the results. The use of CT in assessing body composition has been gaining increasing popularity, and using this methodology it was demonstrated that for a given reported % weight-loss, patients could be losing fat and not muscle, losing muscle but not fat, or indeed gaining in one tissue compartment but with proportionally greater loss in the other (verbal communication Mr Alisdair MacDonald, University of Edinburgh). With the focus of this thesis being on muscle biomarkers and muscle phenotype, the impact of cachexia may have been underestimated by including patients with reasonable weight-loss, but with minimal or no loss of muscle. Assessment of cohorts with radiological evidence of muscle loss (i.e. by CT or MR imaging) may be more useful in future studies. However, as stated in the main introduction to this thesis, there will be few patients with 'healthy' measures of body composition pre-illness which would make the calculation of a %

loss of muscle mass difficult. An alternative proposal (Fearon et al., 2011) would be to combine an absolute measure of muscularity with % weight-loss in order to define a cachectic population. Additionally, a categorical weight-loss cut-off will mean those patients who are pre-cachectic (i.e. on the verge of muscle loss) will be classified as not cachectic. This may not have much bearing on the results of phenotypic studies when early changes in muscle protein pathways may not immediately impact on gross muscle function or mass. However, it would be highly relevant to investigations of intramuscular biomarkers of muscle loss where the sensitivity and specificity of early molecular changes will be compromised by inaccurate classification of cachectic and non-cachectic patients.

Anorexia, systemic inflammation and muscle function

Anorexia is common in cancer cachexia and is likely to exacerbate muscle wasting due to low intake of protein and amino acids. Although none of the patients included in the thesis had supplementary enteral or parenteral feeding, there will likely have been a proportion who had a reduced nutritional intake. It is not clear whether this may have influenced any of the results because an assessment of anorexia was not performed. Accurate measurement of a patient's food or energy intake can be difficult due to the added burden on individuals to write down or weigh foods. Likewise, recall and questionnaires are reliant on memory and are open to misreporting (Wrieden, 2003). However, it would be useful to include some form of assessment in future studies particularly as, for example, the autophagy pathway can be activated by starvation.

Similarly, systemic inflammation is a common finding in cachectic patients and CRP (as a measure of systemic inflammation) was included in the analysis of both the molecular and phenotypic studies. CRP in combination with weight-loss and reduced energy intake has been shown to relate more closely to poorer functional status and prognosis than weight-loss alone (Fearon et al., 2006). However, although it would be interesting to re-examine results using such a definition, the aim of the molecular studies in the current thesis was to look for markers relating to early cachexia. Thus, a definition that classifies a population within the more severe end of the cachexia spectrum would not be informative of early molecular changes that could be used as biomarkers.

The relevance of weight-loss or indeed muscle loss in the definition of cachexia could also be questioned. Whilst many cachectic patients present with weight-loss, if a patient centred approach is used, the key symptoms may be weakness and fatigue. It could then be argued that how much muscle a patient has is not as important as how well the muscle is actually functioning. The disassociation between muscle mass and strength/function was evident in Chapter 7 where a deterioration in muscle quality (strength per unit muscle) was seen in patients with increasing weight-loss. It would therefore be useful to explore definitions of cachexia related to muscle quality or muscle function as a more meaningful patient-centred definition.

2. Study population

Patients with UGI cancer undergoing potentially curative surgery were chosen as the experimental model for this thesis. This was because the incidence of cachexia is

higher in UGI cancer than other tumour types (Dewys et al., 1980) and also to allow access to tissue biopsies at the time of surgery. Using this approach, a unique set of challenges in patient recruitment and investigation were faced.

Challenges with recruitment

Surgery is the only treatment offering potential cure to patients with UGI cancer. Therefore, once a patient has been diagnosed with UGI cancer, they will undergo a barrage of investigations and appointments to assess whether they will be suitable for surgery. For some patients, such as those with obvious metastases, surgery is not an option from the outset. However, for many others several hospital visits are required to assess their cardiovascular fitness, discuss with an oncologist about suitability for neoadjuvant chemotherapy, attend appointments with cancer nurse specialists, along with meeting the surgical team. In parallel with this, patients are coming to terms with their diagnosis of cancer and facing potential mortality. Understandably, it can be a very emotive time for both patients and their families with the added burden of trying to understand their disease and make decisions about treatments. The additional demand of taking part in a research study can therefore appear overwhelming to some patients. Likewise, at a time where hospitals are becoming associated with negative emotions and anxieties, asking patients to attend another appointment for research purposes can be difficult. Several obstacles were also encountered in trying to keep track of where patients were in the treatment pathway and when their date for surgery would be. These factors obviously contributed to some patients being missed, not wishing to participate or only wanting to take part in some of the study (e.g. willing to give a baseline muscle biopsy, but not wishing to

undertake functional assessment). Another challenge of carrying out this study in a tertiary cancer treatment centre was that the hospital received many referrals from surrounding distant geographical areas (e.g. Borders, Fife, Lanarkshire). Approaching such patients for consent prior to their operation, or arranging for functional assessment or MR scans was difficult because they would be admitted the evening before surgery.

However, in spite of these hurdles, the willingness and desire of many patients to help contribute to scientific knowledge without the guarantee of direct personal benefit was striking. Their altruism and generosity was a real example of human goodness that is very much admired.

Challenges with follow up studies

One of the hypotheses of this thesis was 'that it would be possible to undertake a novel longitudinal study of UGI cancer patients undergoing surgery in order to identify a subgroup of patients with progressive cachexia' to allow longitudinal assessment of molecular biomarkers and physiological changes in skeletal muscle. At the outset, we had anticipated recruiting 50 patients at baseline and using MR imaging to measure quadriceps muscle mass, which could then be tracked longitudinally as a direct measure of muscle loss. The intention was to carry out assessments at 6 monthly intervals for 18 months post-operatively and to tie these in with their normal hospital clinic review appointments in order to avoid extra hospital trips and also because there was no travel budget in the supporting grant. It was difficult to stick rigidly to these 6 monthly intervals owing to the fact that patients

not infrequently postponed them to suit holidays or other life events. The time of day in which their appointment was also altered their willingness to stay for a research assessment (i.e. if they lived far away with a long drive ahead, potential to get caught in rush hour, adding cost to their car parking fee at the hospital).

There was also the challenge of selective attrition. At the time of contacting patients for the first follow up appointment at around 6 months, it became evident that several patients had died within this time frame. These patients were presumably the frailer/cachectic patients whereas the fitter patients who did not have progressive disease or cachexia were alive and able to return for follow up assessments. There were also some patients with severe cachexia who were crippled with adverse symptoms (e.g. weakness, fatigue, nausea) along with the sequelae of having been through neoadjuvant chemotherapy and surgery. As such they did not feel able to attend for assessments. Thus, the follow up results were probably less informative about progression of cachexia due to the fallout of potentially the most informative patients. Table 10.1 outlines the numbers of patients aimed to be recruited at each time-interval in comparison with the actual numbers managed for the muscle functional assessments and MR scans.

In order to try and counteract these problems, an additional non-surgical cohort was recruited who had non-resectable malignancy or metastases with the intention that they may be informative by inclusion in the palliative/progressive cachexia cohort. However, this similarly proved a difficult cohort to monitor longitudinally. Patients had reduced life expectancy and thus were unlikely to make it to 6 months.

Table 10.1: Anticipated recruitment and actual number of patients undergoing muscle function and mass assessment

	Baseline	FU1	FU2	FU3
Anticipated	50	35	20	15
Actual function	54	27	18	9
Actual MR imaging	33	21	11	6

The aim was to recruit 50 patients at baseline and follow them up at ~6 month intervals for 18 months. However, patients declined more rapidly than anticipated resulting in smaller numbers at follow-up appointments. Likewise, numbers of patients undergoing muscle mass assessment using MR imaging were less than planned. Abbreviations: FU, follow-up assessment; MR, magnetic resonance.

Furthermore, there was variability in an individual's response to palliative chemo/radiotherapy. For example, one patient with oesophageal cancer undergoing palliative chemo/radiotherapy survived a further 2 years with an initial gain in weight of 20% following recruitment into the palliative group. This made interpretation of the data very difficult due to small numbers and large inter-individual heterogeneity and inclusion of this cohort in the longitudinal study was not felt to be informative.

Heterogeneity of tumour type and operation

In the current thesis, oesophageal, gastric and pancreatic cancer patients were combined for analysis. Whilst all of these tumours are associated with a high incidence of cachexia, it is possible that there are tumour specific differences in the mediators and molecular pathways relevant in muscle wasting, or indeed in the phenotype. For example, patients with oesophageal tumours are more likely to have dysphagia and pancreatic tumours may be more associated with more rapid weight-loss (Dewys et al., 1980) and increased systemic inflammation (Fearon et al., 1999) than other tumour types. Likewise, there may be differences in the impact on patients undergoing oesophageal resection compared with a gastrectomy or Whipples pancreaticoduodenetomy. Such differences in post-operative nutritional status have been reported recently (Carey et al., 2011) and were discussed in Chapter 9. However, the numbers involved are small and further multicentre studies will be required in order to explore the nuances of tumour specific features of cachexia in a more conclusive fashion.

Late recurrence of tumour

At the start of this thesis, the initial supposition was that some of the cancer patients would have recurrent disease post-resection (based on previous projects by members of the research group) within the time frame of the study. Thus a repeat assessment and biopsy would allow longitudinal assessment of a subpopulation with progressive cachexia. However, the patients that did recur either did so early and died before follow up assessment, or remained clinically disease free during the study period and then recurred late after the final 18 month follow-up appointment. Some of this may be due to implementation of better pre-operative staging (e.g. the increasing use of endoscopic USS, PET scan, and higher resolution CT scans) and thus patients undergoing resection were more likely to benefit from the potential of cure. Regardless of the reason, it was not possible to test the initial hypothesis. Rather an alternative model was used to investigate the impact of pre-operative cachexia on recovery post tumour resection as discussed in Chapter 9.

Impact of co-morbidities

The main theme of this thesis was to characterise molecular markers and physiological changes in skeletal muscle in patients with cancer cachexia. However, cachexia can occur in many other chronic diseases such as COPD, cardiac failure, HIV and renal failure. The interaction of various medications with muscle has also been reported (i.e. statins, ACE inhibitors (Parker et al., 2013, Sanders et al., 2005)). Although patients were recruited on the basis of the underlying diagnosis of UGI cancer, many of these patients also had co-morbidities with a reported incidence of cachexia or were on treatments that could potentially affect muscle anabolic or

catabolic pathways. Whilst patients were excluded if they had, for example, uncontrolled diabetes or were taking anabolic stimulants, it was not possible to control for all potential confounders. There is thus a challenge in interpreting such studies to decipher the mechanisms and phenotype specifically related to cancer cachexia. On the other hand, this is the reality of investigating human disease rather than using pre-clinical models where tighter control of confounders can be achieved. Whilst it would be interesting to control for medications and other comorbidities, the heterogeneity amongst patients would make such studies difficult to design and carry out due to the large numbers of patients that would be needed to allow adequate stratification of patients into relevant groups for analysis. Similarly, exclusion of patients taking certain medications or with concomitant diseases would likely result in small numbers and a study that does not relate to everyday clinical practice.

Impact of gender

Many previous studies in cancer cachexia have not taken into account any potential impact of gender. The current thesis has suggested there is sexual dimorphism in the impact of cachexia on muscle function, mass and quality (Chapter 7). However, it was not possible to divide all of the studies in the thesis by gender due to limitations in numbers.

Impact of obesity

The mean pre-illness BMI of all the cancer patients in this thesis was 28.0 (5.2) kg/m^2 (range 18.7-53.2 kg/m^2) and even at the time of cancer diagnosis was 25.7 (4.5) kg/m^2 (range 16.7-42.5 kg/m^2). This reflects both the increasing levels of obesity in

the West, along with obesity itself being a risk factor for cancer. Intuition may suggest that 10% weight-loss in an individual with a starting BMI of 20 kg/m^2 would have a more profound effect than the same weight-loss in an individual with a starting BMI of 35 kg/m^2. However, there is a lack of evidence to either support or refute this. Recent CT studies have added a further layer of complexity with the finding that sarcopenic obesity is an indicator of poor prognosis (Tan et al., 2009). Whether there are differences in tumour biology in obese compared with normal weight individuals is not known and it is equally possible that the drivers of cachexia are distinct in these two groups. It would appear that, at least in patients who are obese, using weight-loss alone as a cut off for cachexia (as in the current thesis) is not going to be sufficient to identify those with pre-existing sarcopenia. In the future, utilisation of CT-based body composition analysis, or indeed varying the definition of cachexia according to pre-illness BMI may be required in studies that recruit obese patients (Martin et al., 2013).

Impact of age

The mean (SD) age of the cancer patients was 65 (10) years, but spanned from 39-88 years. Undoubtedly a proportion of those in the upper age range will have lost muscle as a result of the sarcopenia of ageing. How this might influence the progress of patients with cancer cachexia is not known; i.e. are the effects simply additive, or synergistic. There is a body of opinion that cancer affecting younger individuals has a different pathophysiology with a much stronger genetic component than cancer in older people, which is likely to be more influenced by accumulating environmental

factors. It could therefore be supposed that cancer cachexia has similar age-dependent features but there are, to date, no studies investigating this.

Another challenge faced in this thesis was the recruitment of healthy controls that were age-matched to the cancer patients. Two main sources of control were used. Firstly, volunteers from the community were recruited for the phenotypic studies using adverts on buses and in bowling clubs. These individuals tended to be the healthy elderly. The second cohort, for the biomarker discovery studies, included 'healthy' patients undergoing surgery for benign, non-inflammatory conditions. This population tended to be younger due to the epidemiology of the underlying diseases and also the exclusion of those with co-morbidities, which had increasing incidence with age. These limitations have been discussed in the relevant chapters. The establishment of a collaborative central healthy control database and tissue biobank may be useful in the future to try and overcome such difficulties in recruiting appropriate controls for individual studies that are limited by time.

3. Molecular biomarker studies

The molecular studies (Chapters 3-5) will have many of the same limitations that have been described in previous paragraphs. However, there are also unique challenges in both collecting the muscle biopsies and in the experiments, which will be outlined below.

Collection of muscle biopsies

The first muscle biopsy was collected at the time of surgery after induction of general anaesthesia. All patients had been fasted overnight, but some were admitted to hospital the evening before whilst others attended the hospital on the morning of surgery. In the latter group, it is conceivable that a period of morning activity (or 'exercise') in getting to the hospital would have activated some anabolic pathways in muscles whereas the bedbound, hospitalised patient would not have this stimulus. Some patients would also have high anxiety and stress levels with the thought of impending major surgery. Whether this would result in any meaningful molecular changes at muscle level is unknown, but there is a potential link with increased cortisol levels (classic stress hormone) and muscle catabolism (Ralliere et al., 1997). The specific molecular effects of anaesthesia on human muscle have not been well studied. However, descriptions and investigations of malignant hyperthermia (a pharmocogenetic condition characterised by disorder of skeletal muscle calcium regulation (Hopkins, 2011)) indicate the potential for interaction.

At the follow-up quadriceps biopsy, patients also underwent an overnight fast, but these biopsies were carried out under local anaesthetic and there was no additional stress of forthcoming major surgery. It would be hard to control for these influences, and whether they are relevant requires further investigation, probably in healthy patients in the first instance.

Experimental procedure

One of the characteristics of cachexia is muscle atrophy. In animals, this is predominantly thought to target Type II muscle fibres (Acharyya and Guttridge, 2007). The situation in human cachexia is unclear as there are no studies explicitly investigating muscle histology/fibre type. If the same is true in humans, this raises an intriguing conundrum. In order to extract protein and RNA from muscle biopsies, approximately 20mg of tissue was homogenised for all samples in this thesis. If muscle is atrophying, cutting the same amount from the biopsy will result in a larger number of muscle fibres being included in the cachectic samples compared to those without muscle wasting. Similarly, if the proteins of cachectic muscles that are being degraded relate to myofibrillar rather than sarcoplasmic elements then, in the Western blot experiments, loading the same quantity of protein from these muscles as non-cachectic muscles will result in a potential bias if the biomarker is derived from the sarcoplasmic compartment. Likewise, using a 20mg piece of tissue does not take into account any variability in ratio of Type I:II fibres between individuals. Moreover, cells other than myocytes (e.g. adipocytes, endothelium, fibrocytes, inflammatory cells) are necessarily included in any biopsy. This in turn may confound assessments of individual biomarkers. Perhaps the use of techniques such as laser capture microdissection to ensure equal numbers of fibres of pure myocytes from cachectic and non-cachectic patients may elucidate any potential confounding influence of these factors in the future.

4. Phenotype studies

Functional assessments

Functional measurements were carried out at one point in time. This introduces the potential for results to be influenced by how the patients felt on the day. The measures were also voluntary, and although instruction was given to try as hard as they could, there will undoubtedly be inter-individual variation according to personal motivation. Other research groups have used forced muscle contraction using electric stimulation to overcome this potential problem. However, in the current thesis, it was felt that although it may give a measure of maximal involuntary muscle function, it did not reflect real life and may also have been poorly tolerated in the frailer cancer patients. The longitudinal biomarker study in Chapter 4 utilised the ActivPAL™ monitor. This measures daily activity over several days and thus may be more reflective of patients' true functional levels.

Challenges with MR scanning

The project was based in a busy teaching hospital where the radiology department is pushed to cope with the demands of inpatient and outpatient clinical requests. This meant that on occasion there was not space to accommodate research scans, or that only specific times were available that did not coincide with patient availability. In particular, current cancer treatment target times leave very little scope for flexibility due to impending operation dates and thus a significant proportion of patients were unable to have a pre-operative MR scan. There are also specific contra-indications to MR imaging (i.e. pacemaker, potential metal fragments in orbits, shrapnel), which excluded some patients. In clinical practice, a plain X-ray of the orbits would be

performed to exclude a foreign metal body, but the current research project did not have funding for additional X-rays or the ethics to support exposure to a dose of radiation. Follow-up appointments were more successful as there could be pre-planning and request to the radiology department for MR imaging slots. Although the radiographers were very willing to accommodate as much as possible, there were periods during the study where the MR scanner was being replaced, serviced or had broken down. All these factors meant the numbers who had MR scans were far lower than anticipated and thus the plan to use them to track muscle loss over time had to be re-thought. Part of the solution to this was retrieving CT scans for L3 analysis of muscularity. This resulted in being able to assess muscularity in more patients at baseline, but again longitudinal measures were limited both by lower numbers and by the timing of scans not coinciding with functional assessments.

10.3 ***Future areas of investigation***

Although this thesis has contributed to an improved understanding of the molecular events in skeletal muscle relevant to human cancer cachexia, along with characterisation of the physiological phenotype of skeletal muscle in the cachectic UGI cancer patient, it has also raised many questions and potential avenues for further study. The discovery of a unique gene signature in skeletal muscle along with discovery of β-dystroglycan and CAMK2β as potential biomarkers of cancer cachexia is novel and exciting. However, differences in fold change/protein levels between non-cachectic and cachectic patients were reasonably small and the findings require further validation in larger cohorts before contemplating their clinical application. Furthermore, it would be interesting to investigate potential geographic

differences in the gene profile of cancer cachexia in order to assess the wider applicability of the biomarkers. The studies in this thesis were all in a Caucasian European population and it would be useful to investigate whether the same gene signature of cancer cachexia is relevant in other ethnicities. Likewise, the suggestion that there may be sex differences in response to cancer cachexia in the phenotype studies raises the question of whether there are gender specific molecular pathways in human cancer cachexia. The finding in mice with cancer that cardiac autophagy occurs in a sex specific manner (Cosper and Leinwand, 2011) might suggest that the same may occur in humans, but this will require further exploration in large datasets. There may also be changes that are unique to particular tumour sites. Although all the patients had UGI cancer and the majority were adenocarcinoma, it is possible that there are subtle differences in the phenotype or molecular mechanisms of cancer cachexia according to the underlying diagnosis of gastric, oesophageal or pancreatic cancer.

One of the aims of the thesis was to carry out a novel longitudinal study, but as discussed throughout the thesis, this was not achievable as first thought. In the future, longitudinal studies will undoubtedly be informative given the fact that cachexia is a journey rather than an event. However, careful study design will be required in order to achieve this. It is also apparent that larger numbers of patients will need to be recruited at baseline in order to accommodate the potential fall out in the longitudinal studies. The model used in this thesis was patients with UGI cancer undergoing surgery which would allow more ready access to tissue biopsies with minimal patients discomfort. The percutaneous quadriceps biopsy technique used in this

thesis was tolerated well by the cancer patients undergoing surgery and also at follow up under local anaesthesia. Recruitment of patents who are not undergoing resectional surgery may allow larger numbers to take part in cachexia studies. For example, in an audit of gastro-oesophageal cancer referrals to the regional MDT only 28% of patients were suitable to be assessed for potentially curative surgery. This meant that 72% of patients with UGI malignancies were not approached for recruitment into the studies. It may also be useful to perform follow up assessments at more frequent intervals, and perhaps to utilise more portable equipment which could allow assessments in the patients home environment rather than being confined to the hospital environment. This may improve the recruitment of patients with progressive cachexia and thus the ability to relate the baseline molecular signature to those who go on to develop cachexia rather than differentiating those who are currently cachectic.

The theme of the thesis was intentionally centred around skeletal muscle as this is the key tissue compartment affected by cachexia. However, access to tissue can be associated with complications due to the need for an invasive biopsy. Exploring correlates of the biomarkers that were found to be relevant in muscle (i.e. CAMK2β) in other tissue compartments (i.e. blood/urine) may provide biomarkers that are more easily accessible and applicable to routine clinical practice. Likewise, the observation of increased numbers of lipid droplets in the skeletal muscle of patients with cancer cachexia deserves further investigation into the underlying mechanisms and cross-talk between adipose and muscle compartments in patients with cachexia.

The suggestion that upper and lower limb muscle compartments respond differently to cachexia requires further exploration. A comprehensive assessment of changes and functional measures of all muscle compartments may prove very interesting. For example, if some muscles are more resistant to wasting than others, the reasons for this could be explored and potentially shed light on methods of treating the muscles that are most sensitive. Likewise, further exploration of molecular changes in different muscle compartments could be equally informative.

Although cachexia is characterised by muscle loss, there are no human studies specifically assessing histology/fibre typing of muscles. Likewise, if RNA/protein is degraded in cachexia, it may have implications on molecular studies assessing protein and RNA biomarkers. For example taking a muscle biopsy of the same weight from healthy normal sized fibres compared with atrophying fibres may result in overloading cachectic muscle samples, which will mask potential differences. There is therefore a need to explore this area in more detailed studies.

Another intriguing avenue of investigation would be to focus studies on the non-cachectic patients. Are there reasons why some patients appear to be resistant to muscle wasting despite having the same stage/site of tumour as others who do have profound wasting? Knowledge about muscle molecular pathways and phenotype in these resistant patients could provide very informative in searching for potential treatment targets for cachectic patients. Likewise, with this cohort being less frail, they are more likely to attend follow up assessments and withstand functional

measures rather than the progressive cachexia cohort where many patients become too frail to allow longitudinal assessment.

Potential contribution of thesis to future clinical trial design

The finding that muscle function/mass/quality deteriorates with progressive weight-loss with subsequent impact on QoL begs the question of how to improve or reverse these phenomena. Treatment trials have tended to focus on single pharmacological interventions. However if muscle mass is regulated by diet and exercise, assessment of multimodal treatments focussed on diet/exercise/drug interventions may prove more useful. The present thesis has suggested that a focus on promoting anabolism via protein synthesis would be important in such a multimodal approach. Muscle biomarker research remains at an early stage but could be studied further within the context of such an intervention trial. For palliative patients, the present thesis suggests that the interval over which an intervention should be tested is probably best targeted at 2-3 months rather than 6 months. A post-surgical resection clinical trial model seems too heterogeneous and challenging to recommend at present. From a practical viewpoint, serial assessment of muscle mass is probably best achieved by using diagnostic CT scans rather than specific MR scans. The disadvantage of using such CT images is that lower limb muscles are likely to be more sensitive to change and important differences may be missed. Functional assessment is important and differences between upper and lower limbs suggest that HGD may be less sensitive than lower limb extensor strength and power measures. Furthermore, differences between men and women observed in the present thesis suggest that future intervention trials may have to at least stratify for gender, if not analyse results

separately. Many of the findings of the present thesis have contributed to the design of an international multicentre trial entitled 'Multimodal Exercise, Nutrition and Anti-inflammatory Cachexia Intervention Trial (MENAC)' (Trial No. NCT01419145, (NIH)). The randomised feasibility pilot of this trial has completed and the results of the main trial are anticipated in 2015/16.

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APPENDIX I: Information, screening, consent and questionnaires.

Patient information sheet

THE IDENTIFICATION OF EARLY BIOCHEMICAL AND CLINICAL MARKERS OF CANCER-ASSOCIATED WASTING

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

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

Part 1

What is the purpose of the study?

The purpose of this research study is to find out what causes people who have cancer to become wasted, lose weight and feel tired. This wasting is a significant problem for patients as it causes a reduction in both quality and quantity of life. In order to find out what causes this problem and if there is any way to predict its development, we are asking patients about to undergo surgery for cancer if they would undergo a variety of tests before, during, and 6 months, 12 months and 18 months after their operation. These tests are mainly to measure changes in muscle size, muscle strength and power. We would like to see if any of these changes are related to the activity of certain 'markers' of wasting which we will measure in samples of muscle, tumour, blood and urine. These tests are designed to cause the minimum of discomfort or inconvenience for anyone involved.

Why have I been chosen?

You have been chosen because you will soon be having surgery for cancer. We aim to recruit 50 participants in total.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You will still be free to withdraw at any time in the future and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that you receive.

What will happen to me if I take part?

If you take part, you will be asked to undergo certain tests before your operation, during your operation, and at appointments 6 months, 12 months and 18 months after the operation. The appointments before and after your operation will be timed to coincide with the normal clinic appointments with your surgeon. Therefore, no extra visits to hospital are required if you choose to take part in this study. However, if for any reason we are unable to coincide your study appointments with your normal clinic appointments, we will provide taxi transport to and from the Royal Infirmary of Edinburgh's Clinical Research Facility. The tests will include:

- ***A blood test:*** The amount of blood taken will be 20ml (providing your blood count is normal) and it will be taken from your arm in the usual fashion.

- ***A urine test:*** This will be performed in the usual fashion. The amount of urine taken is 20-30ml.

- ***Tests of muscle strength and power*****:** These tests will involve you sitting in specialised chairs and extending your leg as fast and as hard as you can. These tests will take about 20 minutes.

- ***Test of functional ability (timed up-and-go):*** This test will measure your ability to get up out of a chair and walk a few metres then turn around and go back to your chair. It will only take a few minutes.

- ***MRI scans of your legs and arms:*** These will be carried out in the Department of Radiology and will take approximately 20 minutes to perform. Each scan will involve you lying flat within the scanner although your whole body does not enter the scanner. There are no X-rays or injections involved.

- ***A questionnaire:*** This will take approximately 15 minutes to complete but can be taken home to do. We can provide a stamped-addressed envelope to post it to us if you take it home.

We would initially plan to carry out all of the above tests at one appointment prior to the date of your operation. If, after carrying them out, you found that these tests were acceptable to perform (as we would hope), we would ask you to repeat the same tests at appointments dated 6 months, 12 months and 18 months after your operation.

Apart from the above-described tests, the only other tests will be performed during your operation whilst you are asleep (under general anaesthetic):

- ***A muscle biopsy:*** Whilst you are asleep (under general anaesthetic) during your operation, small muscle samples will be removed from both your abdominal wall and your thigh. The abdominal muscle sample will be pea-sized and will be taken from the incision through which your operation is being performed. The amount of muscle removed from the front of your thigh is even smaller and is taken via a tiny incision (a few millimeters long) through which a needle is introduced. This incision will be closed by paper stitches and will leave a small mark which will fade. If you have a thigh muscle biopsy in the outpatient clinic, this will be done using a technique known as fine needle aspiration. A small needle will be used under suction to aspirate some muscle from the thigh. No stitches will be required after this and it will not be painful as the needle used is very thin.

- ***A fat biopsy*****:** When you are asleep under general anaesthetic, a small piece of fat (again, about the size of a pea) will be taken from just under the skin and also from the fat layer inside of your abdomen.

- ***A tumour biopsy:*** After your tumour has been removed it is normally taken to the Department of Pathology for microscopic examination by a Consultant Pathologist. One of our research team will accompany the tumour specimen to the Department of Pathology, where the Consultant Pathologist will remove small biopsies (a few millimeters) of both the tumour and the surrounding healthy tissue from the specimen and give it to the researcher for analysis.

At one of the appointments dated 6 months, 12 months and 18 months after your operation, we would ask that you give us permission for a further thigh muscle biopsy at that time. This will be performed under a local anaesthetic injection but will otherwise be the same as the one that you have had previously. Obviously we will re-confirm your permission to perform this test at that time.

What do I have to do?

Apart from attendance at the appointments before your operation, and at 6, 12 and 18 months after you operation, no other responsibilities are required from your participation.

What are the possible disadvantages and risks of taking part?

We have taken every step in the design of this study to minimise any possible disadvantages and risks.

Regarding the thigh muscle biopsy, you may experience some mild discomfort and stiffness in the leg following the procedure, but this should wear off after a few hours. Also, approximately 1 in 200 people suffer with bruising following the procedure.

If you have are having a repeat thigh muscle biopsy because you have lost weight following your operation, you may be feeling tired at the time of the procedure. This biopsy will be performed under a local anaesthetic injection. The anesthetic agent may sting for a few seconds as it is being administered.

Regarding the MRI scans, some people may occasionally feel claustrophobic within the scan machine. However, the machine is smaller than other scan machines you may have been in (e.g. CT) and the radiographer remains present with you throughout the scan. The MRI scan does not involve X-rays.

What are the possible benefits of taking part?

We cannot promise that the study will help you, but the information we receive might help improve the treatment of patients with cancer and cancer-associated weight-loss.

What happens when the research study stops?

Following your appointment 18 months after your operation, no further appointments are required. However, if any of your blood, muscle, tumour or urine samples remain, we would ask your permission to store these samples (in anonymised form) in the University of Edinburgh so that we can consider them for use in future research studies that we may carry out (if a local Ethics Committee deems the studies appropriate).

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this, including contact details, is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Part 2

What if relevant new information becomes available?

If any new treatment for cancer or cancer-associated wasting becomes available during the time of the study, it will not be withheld from you because of your participation in this study. Furthermore, if you require any other treatment for cancer during the course of the study (e.g. chemotherapy or radiotherapy), it will not be withheld from you because of your participation in this study.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. However, we would ask your permission to keep in contact with you to monitor your progress. In this way, any information that was collected during the time of your participation in the study may still be used for research purposes. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

What if there is a problem?

- **Complaints:** If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.
- **Harm:** In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed and this is due to someone's negligence, then you may have grounds for a legal action for compensation against the University of Edinburgh but you may have to pay for your legal costs. The normal National Health Service complaints mechanism will still be available to you.

Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. However, we would like to inform your GP of your involvement in this study but we will require your permission to do this. All other information about you which leaves the Royal Infirmary of Edinburgh will have your name and address removed so that you cannot be recognised from it.

Muscle, blood and tumour samples collected during the study may be transferred for the purpose of analysis to associated researchers within and outside the European Economic Area. However, all samples will be anonymised prior to sending and therefore you will not be identifiable.

What will happen to any samples I give?

A portion of the blood samples will be immediately analysed by the Department of Biochemistry at the Royal Infirmary of Edinburgh. The remainder of the blood samples, along with the muscle, tumour and urine samples, will be transferred to the University of Edinburgh for analysis. The only individuals who will have direct access to these samples will be the members of the research team behind this study. The samples will be analysed in the University of Edinburgh by various biochemical techniques in order to measure the levels of certain 'markers' of wasting within the various tissues. Also, small portions of the samples may be sent from the University of Edinburgh to our collaborators at the University of Ohio, USA, for other biochemical analyses which examine different 'markers' of wasting. The samples that are sent to the USA will already be anonymised so that you will not be identifiable from them. Furthermore, all of these samples will be 'used up' in the USA, and therefore, there will be none left over which will require storage overseas.

Following all of these different analyses, if any of the samples remain, we would ask your permission to store these samples (in anonymised form) in the University of Edinburgh so that we can consider them for future research studies (if a local Ethics Committee deems the studies appropriate). Professor Kenneth Fearon, Professor of Surgical Oncology, will act as custodian for any stored samples. The only other individuals who will have direct access to the stored samples will be the members of the research team behind the current study.

Will any genetic tests be done on the samples that I give?

We have no plans to perform genetic analysis within the remit of this current study. However, following this current study, we would ask your permission to store any remaining samples so that we may consider them for use in future research studies that we may carry out (if a local Ethics Committee deems the study appropriate). Future studies could potentially involve genetic analysis, but such studies are at a very early stage of planning and not yet in progress. Any results from future genetic studies will not have any healthcare implications for you and hence we would not normally feed these results back to you.

What will happen to the results of the current research study?

The results of this study will be published in medical journals, reports and textbooks. Results will be made available to study participants through the Cancer Research UK website. You will not be identifiable in any report/publication or report unless you have specifically consented to release such information.

Who is organising and funding the research?

The research is being organised and sponsored by the University of Edinburgh. The research is being funded by Cancer Research UK.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by Lothian Research Ethics Committee. This study has also been reviewed by members of the scientific committee of Cancer Research UK.

Contact details

You may contact me (the main researcher) directly by telephoning 0131 242 6520 for further information at any time. Alternatively, you may contact Mr Rowan Parks, Senior Lecturer and Consultant Surgeon in the Department of Surgery, who is acting as an independent advisor – contact 0131 242 3615.

Many thanks for your time.

Mr. Nathan Stephens
Clinical Research Fellow
Department of Surgery
Royal Infirmary of Edinburgh

Patient consent sheet

CONSENT FORM

THE IDENTIFICATION OF EARLY BIOCHEMICAL AND CLINICAL MARKERS OF CANCER-ASSOCIATED WASTING

1. I agree to take part in the above-titled study. ☐

2. I confirm that I have read and understand the information sheet dated Feb 2008 (version 4.1) for the above study. I have had the opportunity to consider the information and ask questions, and I have had these answered satisfactorily. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from Lothian NHS Trust University Hospitals Division, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to my GP being informed of my participation in the study. ☐

6. I agree to the storage of samples taken during the course of this study so that they may be considered for use in future research studies (pending a favourable ethical opinion by Lothian Local Research Ethics Committee). ☐

7. I agree to the use of samples taken during the course of this study in possible future genetic studies (pending a favourable ethical opinion by Lothian Local Research Ethics Committee). ☐

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Researcher	Date	Signature

QoL questionnaires

1. Karnofsky Performance Score

Which of the nine statements below best describes your present physical activity? Please circle the relevant number.

Your number lies from 80 to 100 only if you are able to carry on normal activity and to work. You require no special care.

100 My activity is normal. I have no complaints and show no evidence of my condition.

90 I am able to carry on normal activity. I have only minor symptoms of my condition.

80 I am able to carry on normal activity but only with effort. I have some minor symptoms of my condition.

Your number lies from 50 to 70 only if you are able to live at home and care for most of your personal needs. You require varying amount of assistance and you are unable to work.

70 I am able to fully care for myself. However, I am unable to carry on normal activity or to do active work.

60 I require occasional assistance, but I am able to care for most of my personal needs.

50 I require considerable assistance and frequent medical care.

Your number lies between 20 and 40 if you are unable to care for yourself. You require the equivalent of institutional or hospital care.

40 I am disabled and require special care and assistance.

30 I am severely disabled. I should be admitted to hospital.

20 I am very sick. I am in hospital. Active supportive treatment is necessary.

2. EORTC QLQ-C30

EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials:
Your birthdate (Day, Month, Year):
Today's date (Day, Month, Year):

	Not at all	A little	Quite a bit	Very much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a long walk?	1	2	3	4
3. Do you have any trouble taking a short walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:	Not at All	A little	Quite a bit	Very much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

During the past	Not at all	A littl	Quite a bit	Ver much
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your family life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your social activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 best applies to you

29. How would you rate your overall health during the past week?

1	2	3	4	5	6	7
Very						Excellent

30. How would you rate your overall quality of life during the past week?

1	2	3	4	5	6	7
Very						Excellent

Healthy elderly screening questionnaire

Exclusion criteria to define "medically stable" elderly subjects

- History of myocardial infarction within the previous 2 years
- Cardiac illness: symptoms of aortic stenosis, acute pericarditis, acute myocariditis, aneurysm, severe angina, clinically significant valvular disease, uncontrolled dysrrhythmia, claudication, within the last 10 years.
- Thrombophlebitis or pulmonary embolus within the previous 2 years
- History of cerebrovascular disease
- Acute febrile illness within the previous 3 months
- Severe airflow obstruction
- Uncontrolled metabolic disease (e.g. thyroid disease)
- Major systemic disease active within the previous 2 years (e.g. cancer, rheumatoid arthritis)
- Significant emotional distress, psychotic illness or depression within the previous 2 years
- Lower limb arthritis, classified by inability to perform maximal contraction of lower limbs without pain
- Lower limb fracture sustained within the previous 2 years; upper limb fracture sustained within the previous 6 months; non-arthroscopic lower limb joint surgery within the previous 2 years
- Any reason for loss of mobility for greater than 1 week within the previous 2 months or greater than 2 weeks within the previous 6 months
- Resting systolic blood pressure.200mmmHg or resting diastolic blood pressure >100mmHg
- Taking beta-blockers or digoxin, or not in sinus rhythm (excluded form ergometry because of difficulty interpreting heart rate)
- On daily analgesics
- Diabetes

Studies of Human Performance in Older People: Health Questionnaire

Name:

Address:

Date of Birth:

Telephone no.:

If the answer is YES to any of the following questions, please give some details

including dates where possible.

Have you any history of heart trouble?
(such as heart attack, angina, valve disease, palpitations, pains in chest, dizzy spells)

Have you any history of problems with blood vessels?
(such as thrombosis, embolus, claudication, aneurysm, dizzy spells, stroke, blood clots)

Have you any history of chest problems?
(bronchitis, asthma or wheezy chest)

Have you ever smoked?
(if YES please state whether you are a current or ex-smoker and how much)

Do you suffer from diabetes?
(if YES please state if insulin dependent)

Have you any history of major illness now or in the last 20 years?
(such as rheumatoid arthritis, blood disorders, cancer)

Have you any history of emotional or psychiatric problems?

Do you suffer from osteoarthritis?
(if YES please state joints affected and indicate mild, moderate or severe and any medication regularly taken)

Have you broken or fractured any bones? If so, when?

Do you have any problems with your bones?
(osteoporosis, loss of height)

Have you any history of back problems? If so, when did they start and do they still affect you in any way?

Have you had any surgery on your joints? If so, when?

Do you suffer from high blood pressure?

Have you had any acute illness in the last six months?
(such as influenza, recurrent sore-throat, bronchitis)

Please state any medication, prescribed or over the counter, regularly taken for any condition

Name of medication How often medication is taken

Have you been in hospital in the last 5 years? If so, why and for how long?

Do you have any physical disabilities?
(such as visual or hearing problems)

Is there any other illness or condition that affects your general health or interferes with your mobility?

Approximately how tall are you?

Approximately how much do you weigh?

Your Doctor's Name:

Your Doctor's Address:

Thank you for completing this questionnaire

APPENDIX II: Patient demographics for the tissue biobank and physiological assessments

Tissue biobank and phenotypic measures

One of the aims of the thesis was to establish a novel biobank of UGI cancer patients. This appendix details all the samples and measurements obtained and the basic demographics of the different cohorts.

Patients undergoing surgery for histologically or clinically suspected UGI cancers were approached for recruitment. 122 participants were recruited to the tissue biobank. This comprised 19 control patients and 103 cancer patients. One control patient did not have tissue biopsies and was thus excluded and a further couple of controls were excluded as detailed below. Post-operatively, 10 cancer patients were excluded due to the pathological examination of the resection specimen not showing a carcinoma. Reasons for exclusion are outlined in Table A1.

This left a total of 16 controls and 93 cancer patients in the tissue biobank.

Table A1: Patients excluded from the tissue biobank.

Initial patient group	Reason for exclusion
Control 1	No tissue biopsies obtained
Control 2	Low BMI, myopathy on pathology
Control 3	Unexplained weight-loss
Cancer Patient 1	Pancreatic neuroendocrine neoplasm
Cancer Patient 2	Oesophageal high grade dysplasia/Barrett's
Cancer Patient 3	Pancreatic neuroendocrine neoplasm
Cancer Patient 4	Pancreatic tubulovillous adenoma
Cancer Patient 5	Pancreatic benign tumour
Cancer Patient 6	Chronic pancreatitis
Cancer Patient 7	Pancreatic serous microcystic adenoma
Cancer Patient 8	Duodenal flat tubular adenoma
Cancer Patient 9	Oesophageal high grade dysplasia/Barrett's
Cancer Patient 10	Pancreatic mucinous tumour

Abbreviations: BMI, body mass index.

Description of cancer patients in the biobank

Tumour site

27 patients had oesophageal cancer, 12 patients had gastro-oesophageal junctional cancer, 15 patients had gastric cancer, 36 patients had pancreatic cancer, 1 patient had duodenal cancer, 1 patient had common bile duct cancer (cholangiocarcinoma) and 1 patient had a small bowel cancer. Within the pancreatic cancer group, there were some patients who had a duodenal or common bile duct cancer invading the pancreas along with one who had a synchronous gallbladder carcinoma.

Histology

The majority of patients had adenocarcinoma (n=81) with 4 of these patients having ACC with other histology also (e.g. IPMN). 5 patients had unspecified carcinoma and 7 patients had squamous cell carcinoma (oesophageal).

Cancer stage

Tumours were reported according to the TNM system and then they were classified into gross cancer stage group according to the UICC criteria. 1 patient had stage 0 cancer, 13 stage 1, 23 stage 2, 37 stage 3 and 19 stage 4 cancer.

Cachexia

When cachexia was defined as pre-operative unintentional weight-loss ≥5%, 50 patients were cachectic and 43 were not cachectic.

Surgery

The majority of patients underwent surgery (n=87). 67 of these patients had what was thought to be a 'curative' resection, and 20 patients had a 'palliative' operation. The latter group were patients who were found to have metastases on opening the abdominal cavity or

where it was not possible to resect the primary tumour (i.e. if invading vessels or extensive adhesions from previous surgery). The types of surgery that were performed were:

Pancreatic cancer: 15 Double bypass (palliative gastrojejunostomy and hepaticojejunostomy), 18 Whipples pancreaticoduodenectomy, 1 Distal pancreatectomy
Gastric cancer: 6 Subtotal gastrectomy, 8 Total gastrectomy, 1 Distal gastrectomy, 1 Proximal gastrectomy
Oesophageal cancer: 29 Ivor Lewis oesophagectomy, 2 Transhiatial oesophagectomy, 2 Thoracoscopic assisted oesophagectomy
Other: 1 Small bowel resection, 3 Laparotomy only (resection abandoned due to distant metastases), 6 patients did not have surgery, but still underwent a muscle biopsy and a blood and urine sample were taken. These patients were thus included in the palliative group.

Basic demographics

Table A2 shows basic demographics for the cancer patients in the biobank.

Tissue samples in the biobank

It was not possible to get all tissue biopsies from every patient. Biopsy samples also varied in size with the result that there was insufficient tissue to divide for protein, RNA and TEM. The biobank was and is an evolving resource. As time passed, more potential uses were thought of and then ethics approval obtained to collect tissue. This resulted in, for example, the patients first entered into the biobank not having fat biopsies.

Tissue biopsy type, preparation and the number of samples for cancer patients are outlined in Table A3 and Table A4.

Table A2: Cancer patient demographics in tissue biobank

Age (years)	65 (10), 39-88
M:F	65:28
BMI baseline (kg/m^2)	25.7 (4.5), 16.7-42.5
Weight-loss (%)	7.7 (9.3), -10.4-46.3
KPS	88.2 (13.2), 50.0-100.0

Results are presented as mean (SD), range, or categorically. Abbreviations: BMI, body mass index; M, male; F, female; KPS, Karnosfky performance score; kg, kilograms; m, metres.

Table A3: Types of tissue in the biobank (cancer patients)

Tissue	Preparation	Number patients
Muscle	Rectus abdominis	87
	Cryo-section	32
	Paraffin for H&E	22
	For Electon Microscopy	23
	Quadriceps	46
Fat	Subcutaneous	68
	Paraffin for H&E	15
	Omentum	51
	Paraffin for H&E	15
Tumour	Tumour	13
	Peri-tumoural normal	6
Urine		81
Blood	Whole blood	67
	Red cells	75
	Plasma	75

Abbreviations: H&E, haematoxylin and eosin stain.

Table A4: Numbers of cancer patients undergoing longitudinal muscle biopsy and blood measures

Tissue type	Baseline	Follow-up	Matched paired samples
Quads biopsy	44	23	21
Blood sample	47	25	25

Description of cancer patients undergoing physiological assessment

This group was the main focus of the physiological studies (Part II) and comprised a subpopulation of the entire cohort. There were 54 cancer patients in total – 35 males and 19 females. Tumour site was: 1 bile duct (cholangiocarcinoma), 9 gastric, 17 oesophageal, 5 OGJ, 21 pancreas and 1 rectum. Tumour gross stage was: 5 stage 1 cancer, 7 stage 2, 28 stage 3, 14 stage 4. 23 patients were cachectic (5% weight-loss cut-off) and 31 patients were not cachectic. Table A5 illustrates the demographics for this cohort.

Longitudinal cohort

Table A6 outlines the numbers of patients undergoing follow up measures. The patients who were not contacted for further follow-up were a small group who subsequently entered a trial involving a potential anabolic agent and thus were not felt appropriate to include in follow up assessment of muscle function and mass. There were also a few patients who had not yet undergone the follow-up appointments at the time of data analysis.

This made the longitudinal analysis difficult as not all had every assessment at each time point. The initial intention of the study was to monitor loss of muscle mass by MR scan. However, of the 33 patients where it was possible to obtain a baseline MR scan, only 12 patients had at least one follow up MR scan to compare to calculate muscle loss. See Chapter 10 for more discussion on these challenges and solutions.

Table A5: Demographics for cohort of cancer patients undergoing muscle function measurements.

Age (years)	65 (11), 39-88
M:F	35:19
BMI baseline	25.2 (4.3), 20.0-40.0
Weight-loss (%)	8.4 (9.4), -10.4-43.8
KPS	82.4 (13.6), 50.0-100.0

Results are presented as mean (SD), range, or categorically. Abbreviations: BMI, body mass index; M, male; F, female; KPS, Karnosfky performance score; kg, kilograms; m, metres.

Table A6: Numbers of cancer patients undergoing each physiological assessment

	Baseline	FU 1	FU 2	FU 3
Total number	54	27	18	9
MR scan	33	21	11	6
IKES	50	27	18	9
LLEP	52	27	18	9
HGD	51	26	17	9
STS	43	25	18	9
TUG	43	25	18	9
QoL	48	25	17	8
ActivPAL™	20	15	10	4
Died	0	14	20	21
Declined FU	0	7	7	9
Not for FU	0	6	6	6
Awaited	0	0	3	9

Abbreviations: FU, follow-up assessment; MR, magnetic resonance; IKES, isometric knee extensor strength; LLEP, lower limb explosive power; HGD, handgrip dynamometry; STS, sit-to-stand time; TUG, 3m timed up-and-go; QoL, quality of life.

Description of control patients in the biobank

18 healthy controls were recruited into the tissue biobank cohort. Table A7 shows some basic demographics for these control patients in the biobank.

All controls underwent surgery. The types of operations were: 7 Incisional hernia repair, 5 Other hernia repair (i.e. inguinal, umbilical), 1 Excision rectal stump, 1 Transduodenal sphincterotomy, 1 Gastrectomy, 2 Cholecystectomy and 1 Open fundoplication

Tissue biopsy type, preparation and the number of samples for control patients are outlined in Table A8.

Table A7: Demographics for control patients undergoing muscle biopsy

Age (year)	58 (16), 28-86
M:F	9:9
BMI baseline (kg/m^2)	27.6 (5.1), 17.9-35.9
Weight-loss (%)	0.0 (0.0), 0.0-0.0
KPS	100.0 (0.0), 100.0-100.0

Results are presented as mean (SD), range or categorically. Abbreviations: BMI, body mass index. M, male; F, female; KPS, Karnosfky performance score; kg, kilograms; m, metres.

Table A8: Types of tissue in the biobank (control patients)

Tissue	Preparation	Number patients
Muscle	Rectus abdominis	16
	Cryo-section	6
	Paraffin for H&E	3
	For Electron Microscopy	7
	Quadriceps	5
Fat	Subcutaneous	9
	Paraffin for H&E	4
	Omentum	7
	Paraffin for H&E	3
Urine		10
Blood	Whole blood	8
	Red cells	8
	Plasma	8

Abbreviations: H&E, haematoxylin and eosin stain.

Description of control patients undergoing physiological assessment

18 healthy older individuals were recruited from the community as controls. Table A9 outlines demographics for this cohort. No patients were knowingly taking anabolic/catabolic agents or hormone replacement therapy at the time of participation in the study. All patients underwent a stringent screening questionnaire to ensure that they were truly 'healthy'. An example of the questionnaire is outlined earlier in the Appendix.

Additional cohorts used

There were supplemental groups added, mainly to increase the numbers in the molecular analysis and reduce the chance of type 2 errors. In the Western blot/qRT-PCR analysis for FOXO, CAMk2, MuRF1, MAFBx, BNIP3, GABARAPL1, n=9 additional cancer patients and 2 additional control patients were included with *Rectus abdominis* biopsies. In the cross-sectional microarray analysis, n=5 additional cancer patients and 1 additional control patients were added. In the TEM analysis, n=1 extra cancer patient was included. These extra biopsies had been collected by a previous research fellow (Mr Richard JE Skipworth) and were used with his permission. The specific demographics of the entire group are outlined and discussed in the relevant Chapters.

Table A10 outlines normal reference ranges for the plasma markers analysed in the thesis.

Table A9: Demographics for control patients undergoing physiological assessments

Age	79 (3), 75-85
M:F	9:9
BMI baseline (kg/m^2)	25.8 (4.0), 18.5-33.9
Weight-loss (%)	0.0 (0.0), 0.0-0.0
KPS	100.0 (0.0), 100.0-100.0

Results are presented as mean (SD), range, or categorically. Abbreviations: BMI, body mass index; M, male; F, female; KPS, Karnosfky performance score; kg, kilograms; m, metres.

Table A10: Normal reference ranges for blood measures

Measure	Units	Range		
		All	Men	Women
CRP	mg/l	0-5		
Albumin	g/l	35-50		
Haemoglobin	g/l		130-180	115-165
Testosterone	nmol/l		10-30	0.4-3.0
Oestradiol	pmol/l		0-160	75-140
LH	U/l		1-9	2-9
FSH	U/l		1-10	3-10
SHBG	nmol/l		6-45	30-120
Insulin	mU/l	n/a		
Cortisol	nmol/l	n/a		

The reference ranges are based upon the values from the hospital laboratories in the Royal Infirmary of Edinburgh. Abbreviations: CRP, C-reactive protein; LH, luteinising hormone; FSH, follicular stimulating hormone; SHBG, sex-hormone binding globulin.

APPENDIX III: Presentations and publications arising from thesis

Presentation of data from this thesis

Oral presentations

Physical functions and sexual dimorphism
NA Stephens
Cancer Cachexia Conference, Boston, USA, September 2012
INVITED LECTURE

Machiavelli and cancer cachexia
NA Stephens
Hospital Grand Rounds
Wishaw General Hospital, February 2012

Surgical resection relinquishes suppression of skeletal muscle turnover in cancer patients
NA Stephens, IJ Gallagher, AJ MacDonald, RJE Skipworth, H Husi, CA Greig, JA Ross, JA Timmons, KCH Fearon
Society of Academic and Research Surgery meeting, Nottingham, Jan 2012

Skeletal muscle Akt in upper gastrointestinal cancer patients and its potential as a biomarker of cachexia
NA Stephens, IJ Gallagher, JA Ross, KCH Fearon
Society of Academic and Research Surgery meeting, Nottingham, Jan 2012

Short-term follow up in patients with upper gastrointestinal cancer undergoing potentially curative resection
NA Stephens, BH Tan, RJE Skipworth, AJ MacDonald, JA Ross, CA Greig, KCH Fearon
School of Surgery Day, University of Edinburgh,
The Royal College of Surgeons of Edinburgh, December 2010
Runner up in **CLINICAL PRIZE** session

Cancer cachexia
NA Stephens
Hospital Grand Rounds
Monklands hospital, Airdrie, September 2010

Reduced muscle quality is observed with progressive weight-loss in patients with cancer
NA Stephens, C Gray, C White, BH Tan, KCH Fearon, CA Greig
School of Surgery Day, University of Edinburgh,
The Royal College of Surgeons of Edinburgh, December 2009

Cancer cachexia – pathway activation in human skeletal muscle
NA Stephens, IJ Gallagher, BH Tan, O Rooyackers, RJE Skipworth, L Lundell, JA Ross, DC Guttridge, JA Timmons, KCH Fearon
School of Surgery Day, University of Edinburgh,
The Royal College of Surgeons of Edinburgh, December 2009

Human cancer cachexia – pathway analysis and identification of novel biomarkers in skeletal muscle
NA Stephens, IJ Gallagher, BH Tan, O Rooyackers, RJE Skipworth, L Lundell, JA Ross, DC Guttridge, JA Timmons, KCH Fearon
Society of Academic and Research Surgery meeting, London, Jan 2010
Presented in **PATEY PRIZE** session
Presentation can be viewed on YouTube (http://youtu.be/F8L_X7uY4k4)

Muscle strength, volume and quality in patients with upper gastrointestinal cancer
NA Stephens, C Gray, C Eeley, BH Tan, KCH Fearon, CA Greig
School of Surgery Day, University of Edinburgh,
The Royal College of Surgeons of Edinburgh, December 2008

An investigation into the role of Forkhead box class O (FOXO) transcription factors in pancreatic and gastro-oesophageal cancer cachexia
R.I. McMurray, N.A. Stephens, K.C.H. Fearon, J.A. Ross
School of Surgery Day, University of Edinburgh,
RCSEd, Edinburgh, November 2007

Poster presentations

Suppression of skeletal muscle turnover in cancer cachexia: evidence from the transcriptome in sequential human muscle biopsies
NA Stephens, IJ Gallagher, AJ MacDonald, RJE Skipworth, H Husi, CA Greig, JA Ross, JA Timmons, KCH Fearon
6th Cachexia Conference, Milan, Italy, Dec 2011

Association of lower limb power output and functional ability in cancer cachexia

NA Stephens, KCH Fearon, CA Greig
6th Cachexia Conference, Milan, Italy, Dec 2011

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