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PHENOTYPES AND GENETIC MARKERS OF CANCER CACHEXIA

Neil Johns

MD Thesis presented to The University of Edinburgh – 2015

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DECLARATION OF ORIGINALITY

I declare that the work described in this thesis was undertaken by me and the thesis was composed and referenced by me personally. This work has not been submitted for any other professional degree or professional qualification.

Where I have collaborated and formed a research group to help with the work, I have directed and made a substantial contribution to the work. Any contribution made by others to the work is acknowledged below.

A tissue bank of patients involved in this thesis was accessed. For the patient's recruited for the work entitled 'clinical classification of cancer cachexia: phenotypic correlates in human skeletal muscle', whole rectus muscle samples were sent to Carsten Jacobi and Shinji Hatakeyama in Switzerland for protein assays and immunohistochemistry.

The systematic review of the genes involved in cancer cachexia was built upon an existing frame work conducted originally by Benjamin Tan. As these genes are still relevant to the genetics of cancer cachexia and come from a specific source there is overlap. The new biology since the previous publication has been added which forms the novel findings of this work.

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2. **Johns N**, Hatakeyama S, Stephens NA, Degen M, Greig C, MacDonald A, Ross JA, Roubenoff R, Glass DJ, Jacobi C, Fearon KCH.
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3. **Johns N**, Stephens NA, Fearon KCH.
   Muscle wasting in cancer
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4. **Johns N,** Stephens NA, and Preston T.
   Muscle protein kinetics in cachexia
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   Review.
   PMID: 23123817

5. **Johns N**, Grieg C, and Fearon KCH.
   Is tissue cross talk important in cancer cachexia?
   Crit Rev Oncog. 2012;17(3):263-76. Review.
   PMID: 22831157

ABBREVIATIONS

^{18}F-FDG	F-fluorodeoxyglucose
ACE	Angiotensin Converting Enzyme
ACSL5	Acyl-CoA Synthetase Long-Chain Family Member 5
ACTN	Alpha-actinin
ACVR2B	Activin Receptor Type-2B
ADCY	Adenylate Cyclase
ADIPOQ	Adiponectin
ADIPOR	Adiponectin Receptor
AEE	Activity Energy Expenditure
AGER	Advanced Glycosylation End Product-Specific Receptor
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALB	Albumin
AMPK	5' AMP-activated protein kinase
ANG	Angiogenin
APC	Adenomatosis Polyposis Coli
APCDD	Adenomatosis Polyposis Coli Down-Regulated
APEH	Acylaminoacyl-Peptide Hydrolase
APOC	Apolipoprotein C
APOE	Apolipoprotein E
APP	Acute Phase Protein
APPR	Acute Phase Protein Response
APR	Acute Phase Response
ARC	Arcuate Nucleus
ASTORIA	Automated Stored Image Analysis
ATG	Autophagy Related
ATGL	Adipose Triglyceride Lipase
ATP	Adenosine-5’-Triphosphate
AU	Arbitrary Units
AZGP1	Alpha-2-Glycoprotein 1
BAT	Brown Adipose Tissue
BCL	B-cell Lymphoma
BMI	Body Mass Index
BNIP3	BCL2/Adenovirus E1B 19kDa Interacting Protein
C-26	Colon-26 Adenocarcinoma Mouse Model
CAM	Cell Adhesion Molecule
CAMK2B	Calcium/Calmodulin-Dependent Protein Kinase II Beta
CCL	Chemokine (C-C motif) Ligand
cDNA	complementary DNA
CHF	Congestive Heart Failure
CHO	Chinese Hamster Ovary

CKD	Chronic Kidney Disease
CLIP	CAP-GLY Domain Containing Linker Protein
CNR	Cannabinoid Receptor
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
COPD	Chronic Obstructive Pulmonary Disease
COX	Cytochrome C Oxidase
CPN1	Carboxypeptidase N polypeptide 1
CRHR	Corticotropin Releasing Hormone Receptor
cRNA	Complementary RNA
CRP	C Reactive Protein
CSA	Cross Sectional Area
CT	Computerised Tomography
CTSZ	Cathepsin Z
CXCR	C-X-C Chemokine Receptor
CYC	Cytochrome C
DCD	Dermcidin
DCTN	Dynactin
DGC	Dystrophin Glycoprotein Complex
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DPT	Dermatopontin
ECM2	Extracellular Matrix
ECOG	Eastern Cooperative Oncology Group
EDL	Extensor Digitorum Longus
EDTA	Ethylenediaminetetraacetic Acid
EIF	Eukaryotic Translation Initiation Factor
eQTL	Expression Quantitative Trait Loci
ERK	Extracellular Signal-Regulated Kinases
ESPEN	European Society of Parentral and Entral Nutrition
FA	Fatty Acid
FABP	Fatty Acid Binding Protein
FBLN1	Fibulin 1
FBXO	Forkhead Box Only
FCSA	Fibre Cross Sectional Area
FD	Fold Difference
FFA	Free Fatty Acid
FFMI	Fat Free Mass Index
FGB	Fibrinogen Beta Chain
FOX	Forkhead box
GABRAPL	Gamma-Aminobutyric Acid Receptor-Associated Protein-Like
GCCR	Generalized Glucocorticoid Resistance
GCDH	Glutaryl-CoA Dehydrogenase

GCKR	Glucokinase Regulator
GDF	Growth / Differentiation Factor
gDNA	Genomic DNA
GDP	Guanosine Diphosphate
GHRL	Ghrelin Hormone Receptor Ligand
GI	Gastrointestinal
GNB	Guanine Nucleotide Binding Protein
GNRH	Gonadotropin-Releasing Hormone
GPCR	G-protein-coupled receptors
GPS	G Protein Pathway Suppressor
GWAS	Genome Wide Association Study
HINT	Histidine Triad Nucleotide
HNF1A	Hepatic Nuclear Factor 1 Alpha
HSL	Hormone-Sensitive Lipase
HSP90	Heat Shock Protein 90
HWE	Hardy-Weinberg equation
HYLS	Hydrolethalus Syndrome
ICAM	Intercellular Adhesion Molecule
ICU	Intensive Care Unit
IFN	Interferon
IFT172	Intraflagellar Transport
IGF	Insulin-Like Growth Factor
IGFBP	Insulin-Like Growth Factor Binding Protein
IgG	Immunoglobulin G
IKKA	Conserved Helix-Loop-Helix Ubiquitous Kinase
IL	Interleukin
INHBC	Inhibin, Beta C
IPA	Ingenuity Pathway Analysis
IRS	Insulin Receptor Substrate
ITGB5	Integrin, Beta 5
JAK	Janus Associated Kinase
JNK	c-Jun NH2-Terminal Kinase
JUND	Jun D Proto-Oncogene
KBTBD5	Kelch Repeat and BTB Domain Containing Protein 5
L3	3rd Lumbar vertebrae
LBM	Lean Body Mass
LEP	Leptin
LEPR	Leptin Receptor
LIPE	Lipase, Hormone-Sensitive (also known as HSL)
LITAF	Lipopolysaccharide-Induced TNF Factor
LLC	Lewis Lung Carcinoma
LM	Low Muscularity
LOXL2	Lysyl Oxidase-Like 2
LPIN	Lipin

LPL	Lipoprotein Lipase
LRRFIP	Leucine Rich Repeat (In FLII) Interacting Protein
LTA	Lymphotoxin Alpha
LTBP	Latent-Transforming Growth Factor Beta-Binding Protein
LY6G5B	Lymphocyte Antigen 6 Complex, Locus G5B
LY96	Lymphocyte Antigen 96
MA	Megestrol Acetate
mAb	Monoclonal Antibody
MAC16	Murine Adenocarcinoma 16 Mouse Model
MAF	Minor Allele Frequency
MAFbx	Muscle-Specific F-box (also known as Atrogin-1)
MAPK	Mitogen Activated Kinase
MBL	Mannose-Binding Lectin
MCR	Melanocortin Receptor
MDT	Multidisciplinary Team
MFAP	Microfibrillar-Associated Protein
MIF	Macrophage Migration Inhibitory Factor
MKNK2	MAP Kinase Interacting Serine/Threonine Kinase 2
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MSH	Melanocyte-Stimulating Hormone
MSTN	Myostatin
MT	Metallothionein
mTOR	Mammalian Target of Rapamycin
MURF-1	Muscle-Specific RING Finger-1
MYF	Myogenic Factor
MyHC	Myosin Heavy Chain
MYOD	Myogenic Differentiation
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NCBI	National Center for Biotechnology Information
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NPY	Neuropeptide Y
NR1H	Nuclear Receptor Subfamily 1, Group H
NR3C1	Nuclear Receptor Subfamily 3, Group C, Member 1
NSCLC	Non-Small Cell Lung Cancer
NTF3	Neurotrophin 3
NTNU	Norwegian University of Science and Technology
NUP	Nucleoporin
OMIM	Online Mendelian Inheritance in Man
P2RY11	Purinergic Receptor P2Y, G-Protein Coupled, 11
PAX	Paired Box

PBMC	Peripheral Blood Mononuclear Cell
PDE	Phosphodiesterase
PDGFR	Platelet-Derived Growth Factor Receptor
PEPCK	Phosphoenolpyruvate Carboxykinase
PET	Positron Emission Tomography
PHLPP	PH Domain And Leucine Rich Repeat Protein Phosphatase
PI3K	Phosphatidylinositol 3-kinase
PIF	Proteolysis Inducing Factor
PIK3	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PLD1	Phospholipase D1
PNPLA2	Patatin-Like Phospholipase Domain Containing 2 (also knonw as ATGL)
POMC	Pro-Opiomelanocortin
PP2C	Phosphatase 2C
PPAR	Peroxisome Proliferator-Activated Receptor
PRKAA	Protein Kinase, AMP-Activated
PS	Performance Status
QoL	Quality of Life
REE	Resting Energy Expenditure
RETN	Resistin
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
rs	refSNP cluster (SNP ID Number)
RTPCR	Reverse Transcription Polymerase Chain Reaction
RUNX	Runt-Related Transcription Factor 1
RXR	Retinoid X Receptor
SCLC	Small Cell Lung Cancer
SD	Standard Deviation
SELP	P-Selectin
SEM	Standard Error of Mean
SGK1	Serine/Threonine-Protein Kinase
SIFT	Sorting Intolerant From Tolerant
SMAD	SMA Mothers Against Decapentaplegic
SMI	Skeletal Muscle Index
SNP	Single Nucleotide Polymorphism
SOD1	Superoxide Dismutase
SPL	Secretory Phospholipase
SPSS	Statistical Package for the Social Sciences
STAT	Signal Transducer and Activator of Transcription
TA	Tibialis Anterior
TAF	TATA Box Binding Protein (TBP)-Associated Factor
TAM	Tumour Associated Macrophage
TEE	Total Energy Expenditure

TFRC	Transferrin Receptor
TG	Triglyceride
TGF	Transforming Growth Factor
T_h	T helper
TIE1	Tyrosine Kinase With Immunoglobulin-Like And EGF-Like Domains
TIRG	Tissue Injury and Repair Group
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TNFRSF	Tumour Necrosis Factor Receptor Superfamily
TOMM40	Translocase of Outer Mitochondrial Membrane 40 Homolog
TRAF6	Tumour Necrosis Factor Receptor (TNFR)-Associated Factor 6
TRHR	Thyrotropin-Releasing Hormone Receptor
TRIM63	Tripartite Motif Containing 63, E3 Ubiquitin Protein Ligase (also known as MURF 1)
TSC	Tuberous Sclerosis
TSH	Thyroid Stimulating Hormone
TTC18	Tetratricopeptide Repeat Domain 18
TWEAK	Tumour Necrosis Factor-Like Weak Inducer of Apoptosis
Ub	Ubiquitin
UCP	Uncoupling Protein
UGI	Upper Gastrointestinal
UK	United Kingdom
UPP	Ubiquitin-Proteasome Pathway
UTR	Untranslated Region
VCAM	Vascular Cell Adhesion Molecule
VDR	Vitamin D Receptor
VHL	Von Hippel–Lindau
WDR20	WD Repeat Domain 20
WL	Weight Loss
ZAG	Zinc-α2-glycoprotein (also known as AZGP1)
χ^2	Chi squared

ABSTRACT

Cancer cachexia is a chronic wasting syndrome characterised by loss of weight, composed principally of muscle and fat. Patients with advanced cachexia demonstrate loss of appetite, early satiety, severe weight loss, weakness, anaemia and fluid retention. Affected individuals are also likely to report/experience decreased quality of life, decreased levels of physical performance, increased levels of fatigue, increased risks of treatment failure (be it chemotherapy, radiotherapy or surgery), increased risks of treatment side effects, and an increased mortality rate. Cachexia is therefore an extremely important, yet often underappreciated cause of cancer patient morbidity and mortality which requires urgent attention. Weight loss is significantly associated with cancer morbidity and mortality. It has been observed that half of all cancer patients experience weight loss and one-third lose more than 5% of their original body weight. Skeletal muscle loss appears to be the most significant event in cachexia and is associated with a poor outcome. However it is not known why some patients with the same tumour lose weight and muscle mass whilst others do not.

The main aim of this thesis was to determine if the genetic makeup of individual patients might contribute to their propensity to lose weight or skeletal muscle. Previous studies had suggested an association between weight loss and SNPs on genes concerned with innate immunity and particularly the cell adhesion molecule P-selectin, however the strength of any gene association study depends on the precision with which it is possible to characterise the phenotype in question. A second aim of this thesis was to explore refining the clinical phenotyping of patients to discriminate those with evidence of muscle fibre atrophy versus those without.

Phenotype

The conventional phenotype for cachexia is weight loss (WL) but it is unknown the extent to which loss of body mass reflects loss of muscle or fat mass. Recent progress in cross sectional imaging analysis means that it is now possible to gain a direct measure of muscle mass from routine diagnostic CT scanning. However, in the absence of a longitudinal series of scans it is not possible to estimate whether low muscularity (LM) is longstanding or not. By combining a measure of active weight loss with low muscularity it was hoped that such a composite measure would reflect actual muscle loss / fibre atrophy. Compared with non-cachectic cancer patients, patients

with LM or LM+>2%WL, mean muscle fibre diameter was reduced by about 25% (p = 0.02 and p = 0.001 respectively). No significant difference in muscle fibre diameter was observed if patients had WL alone. Regardless of classification, there was no difference in fibre number or proportion of fibre type across all myosin heavy chain isoforms. Mean muscle protein content was reduced and the ratio of RNA/DNA decreased in patients with either >5%WL or LM+>2%WL.

These findings support the use of composite measures (WL and LM) to try and identify those patients with evidence of active muscle fibre atrophy. This novel clinical phenotyping provides an accurate method to enable the conduct of candidate gene studies in the investigation of the genetics of cancer cachexia where the primary focus is on muscle wasting rather than overall weight loss.

Genotype

In an ideal world it would be possible to explore the entire genome and look for associations with the different phenotypes of cachexia. However, to do so would require considerable resource in terms of the cost of genome wide analysis and the cost of phenotyping large enough cohorts of patients (3000-10000). To address these issues I therefore adopted a candidate gene approach. A total of 154 genes associated with cancer cachexia were identified and explored for associated polymorphisms. Of these 154 genes, 119 had a combined total of 281 polymorphisms with functional and/or clinical significance in terms of cachexia associated with them. Of these, 80 polymorphisms (in 51 genes) were replicated in more than one study with 24 polymorphisms found to influence two or more hallmarks of cachexia (i.e. inflammation, loss of fat mass and/or lean mass and reduced survival). Such election of candidate genes and polymorphisms is a key element of multigene study design. The systematic review provides a contemporary basis to select genes and/or polymorphisms for further association studies in cancer cachexia, and to develop their potential as susceptibility biomarkers of cachexia.

Phenotype – genotype associations

A total of 1276 patients were recruited, phenotyped and genotyped. There were 545 new patients and 731 patients from a previous study. In our new cohort and in keeping with the previous literature, patients who carried the C allele of the rs6136 SNP in the

SELP gene, were at a reduced risk of developing cachexia defined by WL. This association applied to all degrees of weight loss (>5%, >10% or >15%), and not just at the >10% level as described previously in the literature.

When examining newly identified SNPs in a stage 1 analysis for the weight loss phenotype that included 1276 cancer patients, twelve new candidate SNPs were significant. Six of these SNPs are associated with muscle metabolism in five genes (IGF1, CPN1, FOXO1, FOXO3, and ACVR2B), three are associated with adipose tissue metabolism in two genes (LEPR and TOMM40 (APOE on the reverse strand)), two with corticosteroid signalling in one gene (IFT172 (GCKR on the reverse strand)) and one with the immune response in one gene (TLR4). Two polymorphisms (rs1935949 and rs4946935) in the gene encoding for FOXO3 were consistently associated with WL of increasing severity (>5% and >10%). On the basis that WL is a continuum in the cachectic process, the observation that both SELP and FOXO3 associate with the higher degrees of WL suggests that these genetic signatures may be of particular significance. The role of P-selectin in the genesis of cachexia remains to be determined.

When examining all SNPs in a stage 1 analysis for the LM phenotype, 5 SNPs were associated significantly with the cachexia phenotype: (i) rs4291 in the angiotensin converting enzyme (ACE) gene in chromosome 17; this gene has been associated with muscle function and metabolism; (ii) rs10636 in chromosome 16 in the metallothionein 2a gene; this gene has been shown to be involved in zinc dyshomeostasis which may contribute to cancer cachexia; (iii) rs1190584 in chromosome 14 in the WDR20 gene; this gene encodes a WD repeat-containing protein that functions to preserve and regulate the activity of the USP12-UAF1 deubiquitinating enzyme complex; (iv) rs3856806 in the peroxisome proliferator-activated receptor gamma (PPARG) gene in chromosome 3 which has been demonstrated to be involved in fatty acid and glucose metabolism; and (v) rs3745012 in chromosome 18 in the lipin 2 (LPIN2) gene; this gene represents a candidate gene for human lipodystrophy, characterised by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance.

When examining all SNPs in a stage 1 analysis for the LM +>2%WL phenotype 4 SNPs were associated significantly with the cachexia phenotype. rs12409877 in the leptin receptor (LEPR) located on chromosome 3, LEPR binds leptin and is involved in adipose tissue regulation. rs2268757 located in the activin receptor type-2B

(ACVR2B) gene on chromosome 3, ACVR2B is a high affinity activin type 2 receptor which mediates signalling by a subset of TGF-β family ligands including myostatin, activin, GDF11 and others. SNPs in the tumour necrosis factor (TNF) (rs1799964) and ACE (rs4291) genes were also significantly associated with the phenotype.

Whether genes demonstrating significant associations with the cachexia phenotypes had altered transcript expression in muscle from cancer patients with or without those phenotypes was also investigated. Expression of ACVR2B, FOXO1 and 3, LEPR, PPARG, TLR4, and TOMM40 transcripts was significantly associated with different levels of skeletal muscle index (SMI) or WL ($P<0.05$). Specifically, these were all negatively correlated with muscularity. FOXO1 and 3 and TOMM40 were the only genes significantly correlated with WL; these were correlated negatively with WL.

Of the SNPs found to be significant across the range of phenotypes the majority are exons falling within coding sequences of genes or non-coding regions of genes. Some are introns in the intergenic regions between genes. SNPs may exert differing effects on genes leading to an aberrant gene product. Polymorphisms in promoter regions potentially contribute to differential gene expression, presumably affecting the binding of transcription factors to DNA. Sequence variation in the 5' untranslated region (UTR) could disrupt mRNA translation; mutations in the 3' UTR could affect mRNA through post-transcriptional mechanisms such as splicing, maturation, stability and export. Polymorphisms in intronic regions may result in cis- or trans regulation of genes, unmask cryptic splice sites or promoters leading to alternative transcripts. Synonymous and non-synonymous SNPs in exons could alter protein function or activity and may introduce codon bias contributing to the relative abundance of the proteins, respectively, finally non sense mutations cause a stop altogether in the translation of mRNA. The genomic distribution of SNPs is not homogenous, SNPs usually occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixating the allele of the SNP that constitutes the most favourable genetic adaptation. It has been estimated that 10% of all SNPs in the genome are functional, thereby having the potential of altering some biological process. Whether altering function directly or potentially indirectly all could possibly be used as biomarkers of predisposition to develop cancer cachexia.

The studies presented in this thesis identify new diagnostic criteria that identify patients with evidence of muscle atrophy. They also confirm previous associations with patients who carry the C allele of the rs6136 SNP in the SELP gene are at a

reduced risk of developing cachexia defined by WL and beg the question as to the role of this molecule in cachexia. Whilst achieving these outcomes this thesis also identifies a set of new SNPs that associate with the phenotype which is shown to correlate with actual muscle atrophy.

CHAPTER 1

INTRODUCTION

1.1 Background

Cachexia (from Greek kakos "bad" and hexis "condition") or wasting syndrome is loss of weight, muscle atrophy, fatigue, weakness, and significant loss of appetite in someone who is not actively trying to lose weight. Cachexia has been known and observed for centuries. Hippocrates wrote that “the flesh is consumed and becomes water, the abdomen fills with water, the feet and legs swell, the shoulders, clavicles, chest, and thighs melt away. The illness is fatal.” (1).

Weight loss in cancer patients is due to depletion of both adipose tissue and skeletal muscle mass, while the non-muscle protein compartment is relatively preserved, therefore distinguishing cachexia from simple starvation (2). Cachexia occurs in number of chronic diseases such as cancer, chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), and chronic kidney disease (CKD). It is associated with reduced response to therapies and ultimately accelerated death. Cachexia also impairs quality of life (QoL) and is a significant cause of morbidity and mortality (3).

1.2 Definition of cachexia

Weight loss is significantly associated with cancer morbidity and mortality (4, 5). Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, disease response to therapy, quality of life and duration of survival. Cancer cachexia has been defined in a number of ways (6-10). However, one recent definition described 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 (11).*

Skeletal muscle loss appears to be the most significant event in cachexia and is associated with a poor outcome (11, 12). Death normally ensues when weight loss exceeds 30% (13). It is thought that more than 30% of cancer patients die due to cachexia; that more than 50% of cancer patients die with cachexia being present; and

that the overall mortality rate of patients with cancer cachexia reaches up to 80% (14) (15). Whilst malnutrition is reversible when adequate amounts of food are provided, cachexia is not fully treatable by this approach. Indeed, cachectic patients usually present with progressive weight loss along with body composition alterations and disturbed homeostasis of many body systems, particularly fat tissue and muscle (13, 16).

Systemic inflammation is thought to play an important role in the development of cancer cachexia. Weight loss, muscle atrophy and anorexia have been related, at least in part, to the effects of systemic inflammation (17, 18). C-reactive protein (CRP) is a good surrogate marker of a pro-inflammatory response and has been used to define patients with varying degrees of cachexia (19) (20). The use of CRP has been further verified as it is associated with a poorer prognosis in patients with cancer (21). Markers of systemic inflammation such as CRP have been included in a proposed composite definition of cachexia(22).

1.3 Epidemiology of cancer cachexia

By assessing the degree of weight loss, the incidence of cancer cachexia can be determined. It has been observed that half of all cancer patients experience weight loss and one-third lose more than 5% of their original body weight (23). The most complete study to date was performed in 1980 by Dewys and the Eastern Co-operative Oncology Group (ECOG). It studied the prevalence and prognosis of weight loss in cancer patients who had not yet received chemotherapy (23). The incidence and degree of weight loss over the previous 6 months was recorded in a variety of tumours (Table 1.1). It was observed that the prevalence of weight loss in each tumour type also correlated with magnitude of weight loss.

Of all the cancers observed, patients suffering with Upper GI (gastric and pancreatic) cancer had a high degree of weight loss. Weight loss in pancreatic cancer patients was only analysed over the preceding 2 months, adding further evidence that pancreatic cancer is particularly severe at causing cancer cachexia. Another study demonstrated that 85% of pancreatic cancer patients had lost a median of 14% of their premorbid stable weight at diagnosis and up to 25% at or near time of death (24).

Over this period, LBM fell from 43.4kg to 40.1kg and adipose mass decreased from 12.5kg to 9.6kg (24).

Patients with lung cancer also experience a high degree of cancer associated cachexia. Weight loss at presentation was reported by 59%, 58% and 76% of patients with small cell lung cancer (SCLC), unresectable NSCLC and mesothelioma respectively (25).

Head and neck cancers are another group of patents who experience marked and severe weight loss. Prior to treatment it has been shown that 57% of head and neck cancer patients had lost approximately 10% of their premorbid body weight (26).

The wide variation in weight loss found in the study by Dewys highlights both the importance of the cancer type but also the host's response in determining the amount of weight loss any individual may or may not experience. Table 1.1 demonstrates the varying severity of weight loss experienced by having different types of cancer, it also demonstrates there are a certain percentage of patients suffering from similar cancers who do not experience weight loss. Part of the variation in who will and who will not develop cancer cachexia may be genetically determined and the work in the present thesis sets out to investigate this.

Cancer type	Weight loss in the previous 6 months %			Percentage of patients with any weight loss	Percentage of patients with no weight loss
	0-5	5-10	>10		
Gastric cancer	20	29	38	**87**	**13**
Pancreatic cancer*	29	28	26	**83**	**17**
Non-small cell lung cancer	25	21	15	**61**	**39**
Small cell lung cancer	23	20	14	**57**	**43**
Prostate cancer	28	18	10	**56**	**44**
Colon cancer	26	14	14	**54**	**46**
Unfavourable non-Hodgkin's lymphoma	20	13	15	**48**	**52**
Sarcoma	21	11	7	**40**	**60**
Acute non-lymphocytic leukaemia	27	8	4	**39**	**61**
Breast cancer	22	8	6	**36**	**64**
Favourable non-Hodgkin's lymphoma	14	8	10	**32**	**68**

Table 1.1 - Cancer types with the highest prevalence of cachexia.

Upper GI (gastric and pancreatic) cancer and lung cancer patients, in particular, demonstrate a high prevalence of cachexia. Cachexia is defined as the presence of weight loss over the preceding 6 months *(2 months for patients with pancreatic cancer). Adapted from Dewys *et al*, 1980 (23).

1.4 Staging of cancer cachexia as a spectrum

Cancer cachexia can be classified as a progressive condition with three stages of clinical relevance: pre-cachexia, cachexia, and refractory cachexia (11) Figure 1.1. Each case will be different with not all patients crossing the entire spectrum. Early clinical and metabolic signs can be seen in pre-cachexia (eg, anorexia and impaired glucose tolerance). These often precede substantial involuntary weight loss (ie, ≥5%). Determining which patients will progress is dependent on a number of factors: these include the cancer phenotype and stage; the patient's genotype; the presence of systemic inflammation; low food intake; and lack of response to anticancer therapy. Recently, a panel of experts proposed diagnostic criteria for cancer cachexia as the following: *patients who have more than 5% loss of stable body weight over the past 6 months, or a body mass index (BMI) less than 20 kg/m^2 and ongoing weight loss of more than 2%, or sarcopenia and ongoing weight loss of more than 2% (11).* When considering the severity of cachexia, it is important to consider the proportion of skeletal muscle mass loss and the residual mass still present. For example, a fall of 5 kg/m^2 in BMI from an initial value of 22 has more severe implications for the patient than the same loss from an initial value of 35. Recently, a robust grading system incorporating the independent prognostic significance of both BMI and %WL has been developed (27). Furthermore, a patient with a BMI of 30 and a history of weight loss is more at risk if muscle wasting has occurred (sarcopenic obesity), and less at risk if muscle protein mass remains intact (28). The final stage, refractory cachexia, is a result of very advanced cancer (pre-terminal) or rapidly progressive cancer unresponsive to anticancer therapy. This stage is associated with the presence of patient factors (i.e. bed-rest and immobilisation) that render active management of weight loss no longer possible or appropriate. Refractory cachexia is characterised by a low performance status (WHO score 3 or 4) and a life expectancy of less than 3 months. Artificial nutritional support becomes futile due to the unlikely benefits in outcome. Best supportive care is initiated and the interventions focus typically on alleviating the consequences and complications of cachexia—eg, symptom control (appetite stimulation, management of nausea or eating-related distress of patients and families).

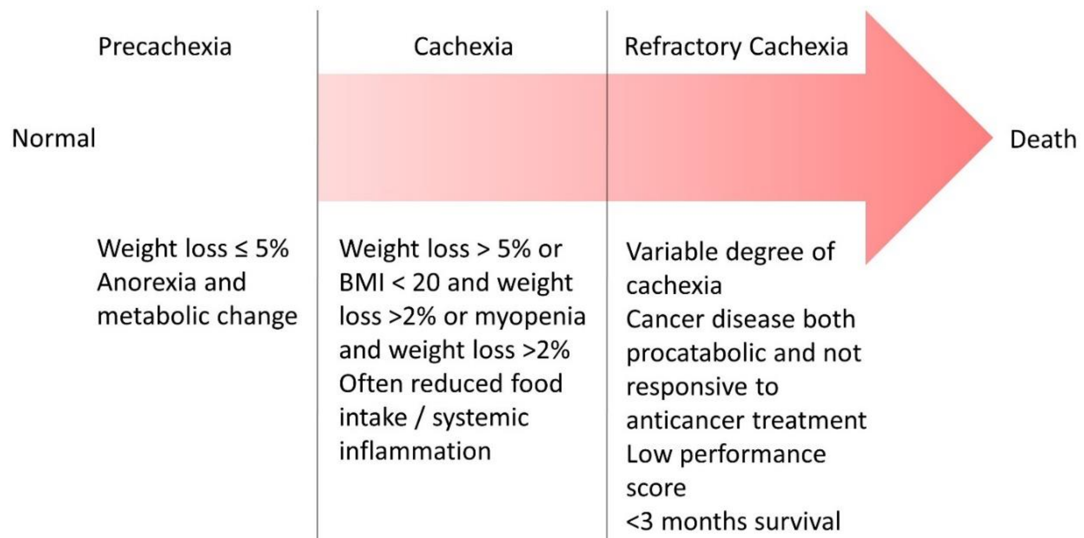


Figure 1.1 - Stages of cancer cachexia

Cachexia represents a spectrum through which not all patients will progress. At present there are no robust biomarkers to identify those pre-cachectic patients who are likely to progress further or the rate at which they will do so. Refractory cachexia is defined essentially on the basis of the patient's clinical characteristics and circumstances. Adapted from Fearon *et al,* 2011 (11). BMI=body-mass index.

1.5 Mechanisms of Tissue Loss

1.5.1 Anorexia / decreased food intake

Body mass is clearly sensitive to food intake. The pathogenesis of cancer anorexia is multifactorial and involves a variety of mechanisms controlling energy homeostasis. The main regulator of the cancer anorexia-cachexia syndrome involves alterations in brain neurochemistry. The hypothalamic melanocortin system does not respond appropriately to peripheral inputs, and its activity switches to the promotion of catabolic stimuli promoting metabolism of carbohydrates, lipids, and proteins in peripheral tissues leading to insulin resistance, increased lipolysis, and accelerated muscle proteolysis (13) (Figure 1.2). Pro-inflammatory cytokines and hypothalamic serotonergic neurons have been implicated in the dysfunction of the hypothalamic melanocortin system (29). There are two main pathways in the control of feeding behaviour: these are the orexigenic neuropeptide Y (NPY), and the anorexigenic (pro-opiomelanocortin) POMC systems (30). Both originate in the hypothalamic arcuate

nucleus (ARC) and extend projections widely over the brain (30). They operate in conjunction with each other and are intrinsically linked. POMC neurons are the source of the potent melanocortin neuropeptides, such as α- melanocyte-stimulating hormone (MSH), which, via interaction with the central MC3/4 - receptors, induce an anorectic state. The role of cytokines in cancer anorexia may be affected through influence on both the NPY and POMC systems. Hypothalamic IL-1 mRNA has been shown to be significantly increased in methylcholanthrene-induced sarcoma bearing rats (31). Levels of IL-1 in cerebrospinal fluid of the same rats are also increased and inversely correlate with energy intake (31). However, injection of an IL-1 receptor antagonist prevents anorexia in the same model (32). In another experimental animal system, injection of IL-1β into the hypothalamus within hours causes significant change in gene expression in skeletal muscle including up regulation of the ubiquitin proteasome pathway (UPP). This clearly demonstrates the potential for neural control of muscle protein synthesis and degradation (33). This mechanism has not been fully explored in cancer cachexia.

Anorexia is a contributor to the weight loss seen in cancer cachexia. Decreased food intake may also result from a number of secondary clinical factors such as disturbances in taste; side effects of chemo / radiotherapy, intestinal obstruction, early satiety, depression, pain, stomatitis, constipation, dyspnoea, and poor dietary habits. Decreased food intake is a common clinical manifestation of cancer occurring in 15% - 40% of patients upon presentation, and rising to nearer 80% in patients with metastatic disease (23). Secondary causes of impaired food intake should be recognised early, because they might prove readily reversible.

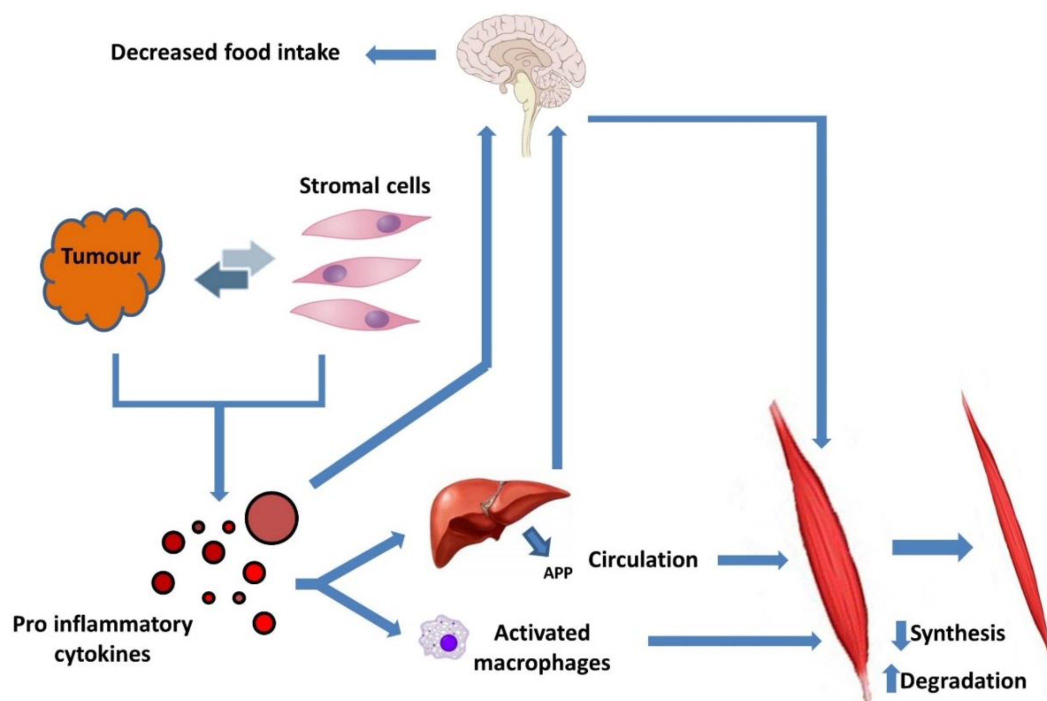


Figure 1.2 – Integrative physiology of cancer cachexia. The cancer is able to activate pro inflammatory, acute phase, and neuro - endocrine responses. The consequences are reduced food intake, and altered protein metabolism.

1.5.2 Increased energy expenditure

There are now many ways to treat cancer involving a mix of surgical options and new systemic anti-tumour therapy. To enable tolerance of such treatments, patients have to maintain an adequate energy and protein balance. Total energy expenditure (TEE) is comprised of resting energy expenditure (REE), diet-induced energy expenditure (DEE), and the energy cost of physical activity (AEE). Approximately half of cancer patients are hypermetabolic, and weight-stable patients tend to have a lower REE (34). In patients with increased levels of inflammatory cytokines, resting energy expenditure (REE) was found to be significantly higher (35). These cytokines cause a cascade of cellular signalling which potentiates the acute phase response (APR). The mediators of the APR initiate a signalling cascade in the in liver altering protein synthesis, which shifts from production of albumin to acute phase proteins (APP), such as C-reactive protein (CRP), fibrinogen, serum amyloid A, and α-1 antitrypsin, in response to an insult such as inflammation. Hypoalbuminaemia is a key clinical

feature of cancer cachexia. The mechanisms by which this is thought to happen are an increase in the synthesis of positive reactants (36) coupled with an increased transcapillary escape rate for albumin (37). During periods of inflammation/injury the metabolic response of the body is to prioritise hepatic export protein synthesis by mobilising protein from skeletal muscle stores. The amino acids available from skeletal muscle breakdown differ from those that constitute acute phase proteins and therefore this may increase the need for degradation of skeletal muscle especially in times of low food intake (38). This is how an APR may contribute to excessive muscle wasting in semi staving, anorexic cancer patients.

The APR is thought to be mainly regulated by IL-6. The APR is linked to the loss of body mass in patients with lung and gastrointestinal cancer (39), and in patients with non- small cell lung cancer (NSCLC), serum levels of IL-6 were found to correlate with concentrations of circulating CRP (40). Hypermetabolism has been shown to be related to systemic inflammation (35) and or the presence of an elevated adrenergic state (41). Both of these are controlled centrally via pathways such as the melanocortin type 4 receptor (42).

Futile substrate cycles may also contribute to hypermetabolism. Despite adequate oxygen supply, most cancer cells use glycolysis as the principal method to generate ATP, and this phenomenon is called the Warburg effect (43). Such high glucose turnover, is useful in clinical practice as it forms the basis of deoxyglucose positron emission transition (PET) scanning to detect local or advanced disease. Increased glucose uptake and glycolysis has been ascribed to overexpression of various oncogenes (e.g., c-Myc, Ras, Akt), or loss of tumour suppressors (p53 and Vhl) (44, 45), abnormal mitochondrial function (46), overexpression of type II hexokinase due to gene demethylation (47), or the hypoxic environment of the tumour and hypoxia-inducible factor 1 effects on glucose metabolism. The Cori cycle converts excess lactate produced by the tumour to glucose in the liver via pathways that lead to increased consumption of ATP. Cori cycle activity in weight-losing cancer patients has been studied and shown to be upregulated (48), however, there is a poor correlation between tumour burden and an increased lactate production (49). Overall consumption of glucose via gluconeogenesis and glycogenolysis has been shown to be increased in weight-losing cancer patients (50) and it has been suggested that this may be responsible for 40% of the increase in energy expenditure in metastatic cancer patients (51).

Brown adipose tissue (BAT) activation is thought to occur in the cachexic process and contribute to hypermetabolism. Adaptive thermogenesis is a unique process whereby increased expression of UCP 1 in the mitochondria of BAT leads to dissipation of the proton gradient so that oxygen consumption is no longer coupled to ATP synthesis and heat is generated resulting in a hypermetabolic state (52, 53). F-fluorodeoxyglucose (^{18}F-FDG) PET-CT led to the idea that BAT activity was increased in patients with cancer. The existence of functional BAT was discovered by studying areas of increased metabolism and glucose uptake with ^{18}FDG on the PET in areas that were unmistakably fat tissue on the basis of Hounsfield units on the CT (54). FDG specifically accumulates in BAT in proportion to the expression of UCP1 that is an exclusive constituent of BAT (55, 56) and BAT activity as assessed by the uptake of ^{18}F-FDG is positively correlated to a cold-induced rise in energy expenditure (57). It has been shown that levels of BAT are greatly reduced in obese individuals (53), this coupled with the idea that BAT contributes to increased levels of energy expenditure has lead it to be a target for the treatment of obesity (58). With regards to its influence in cancer cachexia, three retrospective studies addressed a possible relationship of BAT ^{18}F-FDG activity with cancer, however none of these observed a higher incidence of BAT activity in active and/or PET-positive cancer patients (55, 59, 60). Another study assessed BAT activity from guanosine diphosphate (GDP) binding to mitochondria prepared from perioperative samples. This was found to be elevated in children with malignancy compared with a control group undergoing renal surgery (61). A further study showed that cancer patients had higher metabolic activity of identified BAT on FDG-PET. More active neoplastic status was associated with more vigorous activity of BAT (62). All these studies have focused on the glucose uptake contributing to BAT activity, however fatty acids contribute up to 90% of overall substrate use (63). One study in healthy individuals demonstrated increased uptake by BAT under cold-stressed conditions (64), no studies in cancer cachexia have to date been performed. To further explore the link between BAT and cancer cachexia, studies should focus on the question whether an abnormal thermoregulatory response exists in cancer cachexia, and the quantitative significance of BAT activity for increased energy expenditure and body weight loss in humans.

1.5.3 Other aspects of abnormal metabolism associated with cachexia

1.5.3.1 Altered lipid metabolism

Adipose tissue loss is seen at a greater rate in cancer cachexia than muscle loss (65). This is due to a combination of decreased food intake and increased inflammatory cytokines that either inhibit lipogenesis or promote lipolysis. Adipose tissue loss in cancer cachexia is thought to occur mainly as a result of increased lipolysis (66, 67). The triglyceride component of the lipid is broken down to form free fatty acids and glycerol, which are then released into the circulation. The main enzymes which process this breakdown are hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (68). HSL, ATGL, plasma FFA, and glycerol all increase in patients suffering from cancer cachexia (67). The excess breakdown may be coupled with a decrease in lipogenesis, this leads to a net decrease of adipose tissue resulting in the observed overall loss.

1.5.3.2 Altered protein metabolism

Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients and is associated with a poor outcome (11, 12, 69). In healthy adults, skeletal muscle mass is maintained by nutritional intake and physical activity, reflecting a dynamic balance between protein synthesis and degradation. A predominance of either will result in muscle hypertrophy or atrophy. Even small changes in protein synthesis or degradation will lead to large protein deficits over time due to the continuous process of protein turnover. The myofibril is the base unit of muscle comprising thick (predominantly myosin) and thin (predominantly actin) filaments. Myofibrillar proteins account for more than 50% of protein in skeletal muscle and are integral to muscle contraction and function. The preservation or loss of these proteins will dictate the clinical symptoms and there is some evidence that they are targeted selectively in cancer cachexia (70, 71).

1.5.3.3 Muscle protein kinetics in the healthy adult

Insulin is the most important endocrine factor controlling skeletal muscle protein synthesis in the active, well-fed, healthy adult. Reduced peripheral insulin sensitivity

is described in terms of reduced glucose clearance by peripheral tissues, predominantly skeletal muscle. Glucose uptake and amino acid uptake are both under insulin control and impaired muscle protein synthesis can accompany reduced insulin sensitivity. Normalisation of peripheral insulin sensitivity in subjects whom which it is reduced may improve skeletal muscle protein synthesis.

1.5.3.4 Muscle protein kinetics in cancer cachexia

In cachexia, there is ongoing debate as to whether a reduction in protein synthesis, an increase in protein degradation or a combination of both is more relevant. Although evidence continues to emerge, it would appear that many of the relevant pathways are influenced by the presence of systemic inflammation (21, 72, 73). Inflammatory-mediated signalling may limit muscle protein synthesis by several mechanisms. TNF-α can activate the transcription factor NF-kB, which inhibits the synthesis of the muscle specific transcription factor MyoD, thereby inhibiting differentiation (74), the TGF-β family members induce muscle wasting downstream of SMAD activation (75), and IL-6 activates signalling by binding to ligand-specific receptors either in soluble or membrane-bound forms to induce the signal transducers and activators of transcription-1 and -3 (STAT1/3), ERK and PI3K-AKT pathways (76, 77). The ERK pathway has been previously implicated in cancer cachexia and muscle wasting (78) and the AKT pathway is primarily responsible for anabolism in skeletal muscle (79). Recently a study used a novel technique to measure habitual myofibrillar protein synthesis in patients with cancer compared with healthy controls. Contrary to previous studies, there was no evidence of suppression of myofibrillar protein synthesis in patients with cancer cachexia. This finding implies a small increase in muscle breakdown may account for muscle wasting (80).

1.5.3.5 Proteolytic pathways

Many research groups have focused investigation on mechanisms responsible for skeletal muscle protein degradation in cancer cachexia. Although there are four mammalian proteolytic enzyme systems (the proteasome, lysosomal/autophagy pathway, caspases and calpains), it is the proteasome and lysosomal pathways which have received most attention.

The majority of signalling pathways contributing to muscle atrophy in pre-clinical models are mediated through activation of the ubiquitin-proteasome proteolytic pathway (UPP) (81, 82). The muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1 are up regulated in animal models of atrophy (83, 84), and MuRF1 selectively targets the myofibrillar protein myosin heavy chain resulting in muscle wasting (70, 71). However, the role of the E3 ligases in human cachexia is less well defined. Studies including patients following bed rest, limb amputation for vascular disease, limb immobilisation, COPD, amyotrophic lateral sclerosis and ageing demonstrate both increased and decreased expression of MuRF1 and MAFBx (85-88) and evidence for an increase in human cancer cachexia is lacking (89). There is also data that suggests dissociation between protein dynamics *in vivo* and activation of UPP signalling in human skeletal muscle (90).

Upstream of the E3 ligases, the insulin regulated transcription factors FOXO1 and FOXO3a have been shown to increase expression of MuRF1 and MAFbx in animal models of cachexia (91, 92). Furthermore in both myotube and mouse models of muscle atrophy, several autophagy genes that are upregulated appear to be under control of FOXO3 (91-94), linking the autophagy and proteasomal systems. This makes the FOXO transcription factors attractive therapeutic targets and indeed, early evidence of FOXO inhibition in pre-clinical models is promising (95).

In cancer patients there are few studies relating to the role of the autophagy pathway in muscle wasting. Increased cathepsin D and acid phosphatase activity has been demonstrated in patients with varying tumour types and degrees of weight loss (96) and lung cancer patients undergoing resection were shown to have increased levels of cathepsin B (97). Likewise, expression of the autophagy-related genes GABARAPL1 and BNIP3 has been associated with the presence of systemic inflammation and/or weight loss (89), suggesting a potential role in the development of human cancer cachexia.

1.6 Mediators of Tissue Loss

Cancer cachexia is brought about by a complex interaction between the tumour and the host’s mechanisms responsible for processes including; regulation of body composition (adipose and muscle mass), food intake, and energy expenditure.

Alterations in cytokine concentrations, neuroendocrine activation, and production of cachectic factors by the tumour lead to imbalances of these processes and promote cachexia resulting in a number of metabolic changes that are often characterized by negative energy balance, increased thermogenesis and anorexia (98) (Figure 1.3).

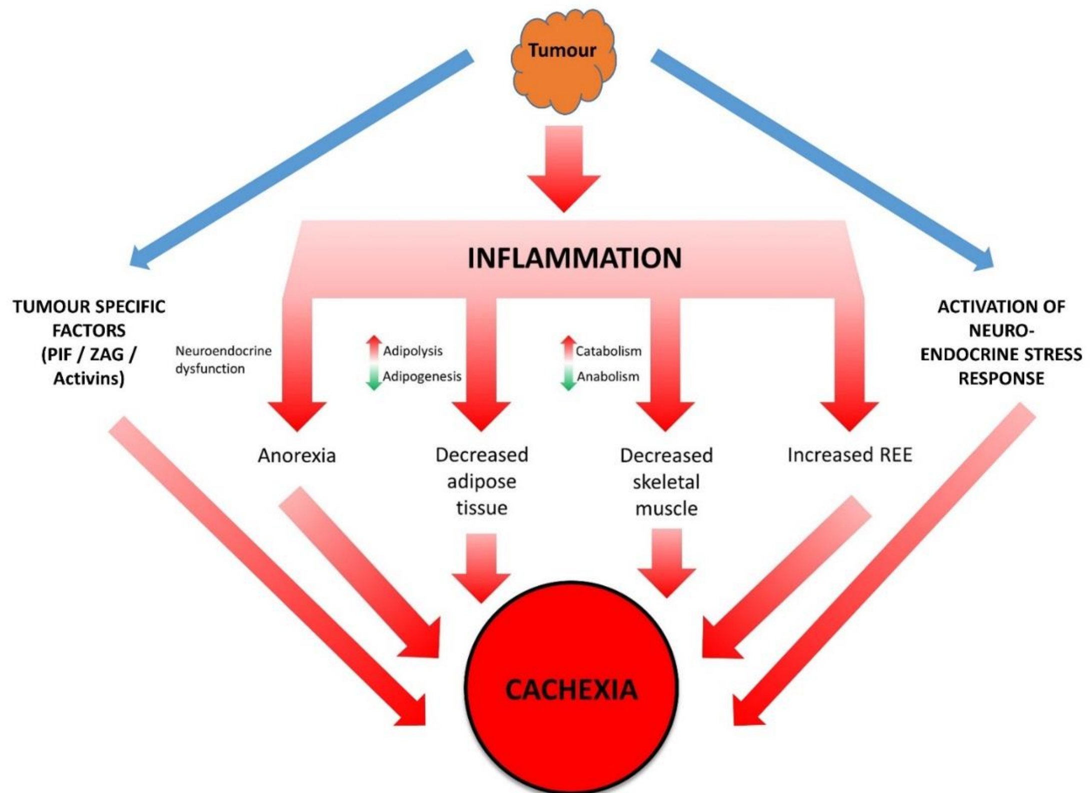


Figure 1.3 - Proposed mediators and mechanisms implicated in cachexia

1.6.1 Changes in muscle and adipose tissue mass

1.6.1.1 Adipokines

Adipose tissue, like skeletal muscle is an active metabolic and endocrine organ. A number of inflammatory cytokines secreted by adipose tissue have been shown with varying effect to influence the development of diseases such as insulin resistance, diabetes, and cancer cachexia by acting on muscle and fat metabolism. Adipokines, include IL-6, TNF-α, IL-1, leptin, zinc-α-2-glycoprotein (ZAG) [other name: Lipid Mobilising Factor (LMF)], and resistin (99). The catabolic effects of cytokines on adipose tissue triglyceride metabolism have been proposed as a contributor to the development of cachexia (100).

IL-6 secretion by adipose tissue appears to play a different role compared with its secretion by skeletal muscle (Figure 1.4). In adipose tissue, IL-6 has been shown to be up regulated in insulin resistant and obese patients (5). Visceral adipose tissue secretes more IL-6 than subcutaneous adipose tissue and is the main source of adipose tissue secreted IL-6. Higher concentrations of IL-6 in the obese population are thought to cause hepatic CRP production, a major risk index for cardiovascular disease (101). The IL-6 receptor signals via the JAK/STATs (Janus kinases/signal transducers and activators of transcription) signal transduction pathway. Downstream activation results in STAT phosphorylation and translocation to the nucleus to regulate genetic transcription (102). How IL-6 affects insulin action is not well established. However, it is widely accepted this adipokine is associated with insulin resistance in the obese patient (5, 103). Il-6 plays an established role in cancer cachexia, circulating levels of IL-6 have been shown to correlate with weight loss in cancer patients, and importantly, IL-6 levels correlate with reduced survival (104, 105). Gain- and loss-of-function experiments in tumour bearing mice also support the requirement of IL-6 in regulating cachexia (106-108).

TNF-α has been shown to play a role in both animal models and humans in insulin resistance (109). TNF-α has a bearing on lipid metabolism in cancer cachexia via the following ways. It regulates cholesterol metabolism and other adipocyte-derived adipokines, suppresses free fatty acid (FFA) uptake and promotion of lipogenesis, as well as inducing lipolysis. Finally it is able to inhibit lipid-metabolism-related enzyme activity (110). TNF-α has been shown to initially have a protective role to try and limit adipose tissue mass by stimulating lipolysis and decreasing LPL expression and activity (111). TNF-α also has a role in the inhibition of adipose hypertrophy (112) possibly via paracrine signalling. TNF-α plays an established role in cancer cachexia, it inhibits both adipocyte and skeletal myocyte differentiation (113, 114) and contributes to insulin resistance in part by impinging on the insulin signalling pathway (115). Addition of TNF-α is sufficient to promote atrophy in cultured myotubes, resulting from the induction of E3 ligase genes that mediate the breakdown of myofibrillar proteins by the ubiquitin- proteasome pathway (116-119).

Leptin is produced by adipose tissue and acts on the hypothalamus to regulate the amount of energy stored in fat via its influence on appetite. There is little doubt this adipokine has a positive association with BMI (120, 121). Reduction of circulating leptin has also been markedly shown as a result of bariatric surgery (122). Studies of

leptin deficient mice (ob/ob), have demonstrated this adipokine is fundamental in the control of body weight and lipid metabolism (123). Experimental administration of leptin has been shown to reduce food intake and promote weight loss (124). Physiologically, leptin is thought to protect adipocytes from lipotoxicity by inhibiting lipogenesis and increasing fatty acid oxidation (124). These studies highlight the role of leptin in regulation of body weight. Furthermore, leptin deficient ob/ob mice have been shown to develop obesity due to lack of appetite suppression. Pathologically, leptin may also act as an adipokine potentiating the chronic inflammation seen in obesity and insulin resistance. Studies have shown hyperleptinemia is associated with a chronic inflammatory state in the non-obese (125). Although leptin reduces appetite, levels tend to be exceptionally high in the obese implying leptin resistance (126, 127).

Adiponectin is produced by the adipocyte and has been shown to increase insulin sensitivity. Unlike other adipokines associated with chronic inflammation, adiponectin is inversely related to insulin resistance and BMI. It appears to have protective metabolic and anti-inflammatory properties (128).

ZAG is involved in the specific mobilisation of adipose tissue and increased oxidation of released fatty acids (possibly via induction of uncoupling protein (UCP) expression). ZAG mobilises fat via the β3 – adrenergic receptor. It is produced by both host and certain tumours. Adipose tissue secretes large amounts of ZAG, and this is accelerated in patients with cancer cachexia and correlates with reported weight loss (129).

1.6.1.2 Myokines

Skeletal muscle, like adipose tissue is now widely viewed as an endocrine organ, with many studies demonstrating that cytokines produced by muscle fibres exert paracrine and endocrine effects; such cytokines (IL-6, IL-15, and TNF-α) have been labelled myokines (5) and exert metabolic effects on a number of other tissues including the liver and adipose tissue.

IL-6 secretion via skeletal muscle has been shown to increase during exercise when insulin action is enhanced (Figure 1.4). It is proposed that IL-6 acts both locally via activation of AMPK in response to muscle contraction but also has endocrine functions. Intramuscular IL-6 expression is regulated by a network of signalling cascades that are likely to involve cross-talk between the $Ca2+$/NFAT and

glycogen/p38 MAPK pathways. Further studies suggest that IL-6 derived from muscle mediates anti-inflammatory effects and IL-6 can inhibit TNF production and stimulate synthesis of cytokine antagonists such as IL-1 receptor antagonist (IL-1ra) and IL-10 (5). This may account for IL-6 being both primarily involved not only in local glucose uptake but also in adipose tissue lipolysis. IL-6 is widely known to act as both a pro and anti – inflammatory cytokine. This is determined mainly by concentration and which tissue it is acting on, whether muscle, adipose tissue or immune cell (130). In addition, a recent study found that skeletal muscle protein synthesis was dramatically reduced by relatively low-dose IL-6 infusion in humans (131).

IL-15 is an important myokine in the regulation of skeletal muscle (132). IL-15 has a role in the differentiation of myocytes and the ability of muscle fibres to increase the density of contractile proteins (133). This is confirmed with mouse models where skeletal myoblast differentiation occurs under certain conditions in response to IL-15 (134). Mechanisms include the transcription factor PPAR-ɣ activating protein synthesis (135). IL-15 has also been shown to regulate muscle mass in response to resistance exercise training in humans. A recent study proposed that IL-15 is predominantly expressed by MyHC II skeletal muscle fibres, and that resistance exercise regulates IL-15 expression in muscle (136) IL-15 mRNA level was enhanced in skeletal muscles dominated by MyHC II fibres and resistance exercise induced increased muscular IL-15 mRNA levels. IL-15 mRNA levels in skeletal muscle were not paralleled by similar changes in muscular IL-15 protein expression suggesting that muscle IL-15 may exist in a translationally inactive pool (136). IL-15 has also been proposed to play a role in the prevention of diabetes by modulating glucose uptake in skeletal muscle (137). A recent study analysed the effects of IL-15 on glucose metabolism in skeletal muscle. A single dose of IL-15 resulted in a significant increase in glucose uptake in human skeletal muscle, animal models, and in C2C12 cells (137).

TNF-α, has been shown to be released by both adipose tissue and muscle. When acting on muscle, TNF-α has been shown to play a role in muscle protein degradation, it has also been shown to induce muscle cell apoptosis (138). TNF-α can activate the transcription factor NF-kB, which inhibits the synthesis of the muscle specific transcription factor MyoD, thereby inhibiting differentiation (74). Release of TNF-α may augment skeletal muscle insulin resistance as well as signalling and potentiating a similar response in adipose tissue. Preliminary reports have described marked elevations in myostatin (a negative regulator of muscle mass that inhibits myogenic

proliferation and differentiation) in cachectic cancer patients. Animal models suggest that the inflammatory cytokine TNF-α is at least partially responsible for increased myostatin (139). TNF-α is also known to negatively influence the anabolic mTOR signaling pathway, which is a major mediator of anabolic responses in skeletal muscle (140).

Inflammatory stimulation activates pathways associated with muscle protein breakdown (141). In murine models of cancer cachexia, the ubiquitin proteasome pathway is greatly influenced (82). Many of these effects are thought to occur through activation of NF-kB by upstream factors such as TNF-α (142). NF-kB stimulates transcription of the ubiquitin E3 ligase muscle ring finger (MuRF)-1, which is known to positively regulate activity of the ubiquitin proteasome pathway (143).

The interplay of anabolic and catabolic hormones has a distinct role in the development of skeletal muscle wasting. The catabolic molecules myostatin and activin are two members of the TGF-β superfamily that play an important role in growth and are thought to be responsible for the development of cachexia in mice (144). In mice, both molecules bind to activin receptor type-2B (ACVRIIB), a receptor in muscle, to initiate a signalling cascade leading to increased expression of MuRF1 and MAFbx/Atrogin-1 and subsequent degradation of myofibrillar proteins (79). In mice bearing the C26 tumour, administration of a soluble ACVRIIB decoy receptor resulted not only in reversal of muscle wasting but an increase in survival (145).

1.6.1.3 Adipose – muscle cross talk

IL-6 has a different role to play in exercise compared with insulin resistance / diabetes. Chronically elevated IL-6 levels lead to inappropriate hyperinsulinaemia, reduced body mass, and impaired insulin – stimulated glucose uptake in skeletal muscle (146). Conversely, IL-6 knockout mice develop obesity and insulin resistance, providing evidence against a causative effect of IL-6 in insulin resistance (147). Clearly IL-6 has numerous roles to play depending on the clinical context, one proposal is that after the peak of IL-6 after exercise, baseline levels return to lower than original and muscle based receptors are down regulated (148). Thus changes in IL-6 levels appear to signify a change between a physiological response and a pathophysiological one.

The myokine IL-15 is an anabolic factor involved in the development of skeletal muscle, it has also been shown to play a part in lipid metabolism (149). In a study by

Nielsen and co-workers, a negative association between IL-15 concentration and trunk fat mass, but not limb fat mass following exercise was observed. IL-15 secretion from muscle can reduce visceral fat without any change to muscle mass demonstrating a potent endocrine function (132). Argiles and co-workers have shown IL-15 acting not just on adipose tissue but on endothelial cells, the gastrointestinal tract, and the liver (150). IL-15 could have therapeutic roles in the treatment of a number of diseases. In obesity it has been shown to have an antiadipogenic effect and could be used to prevent lipogenesis. Animal models receiving IL-15 demonstrated a 33% decrease of white adipose tissue mass. This is achieved through a reduction of lipogenesis coupled with a decreased uptake of VLDL triacylglycerol through lipoprotein lipase (LPL) (151). IL-15 facilitates glucose uptake by insulin sensitive tissues such as skeletal muscle (150). Argiles and co-workers propose a reciprocal control between adipose and skeletal muscle size. They propose that IL-15 is released from skeletal muscle with the aim of controlling fat deposition and, thus, adipose tissue growth and mass. The anabolic effects of IL-15 have also been shown in animal models (151). In rats bearing the Yoshida AH-130 ascites hepatoma, IL-15 treatment partly inhibited skeletal muscle wasting by decreasing protein degradation rates to values even lower than those observed in non-tumour-bearing animals. These alterations in protein breakdown rates were associated with an inhibition of the ATP-ubiquitin-dependent proteolytic pathway. This opens the potential for IL-15 to be used as a therapeutic in wasting diseases such as cancer cachexia.

TNF-α has been shown to play a role in both animal models and humans with insulin resistance (109). TNF-α may initially have a protective role and limit adipose tissue mass by stimulating lipolysis and decreasing LPL expression and activity (111). TNF-α also acts to inhibit adipose hypertrophy (112) possibly via paracrine signalling. This however, results in an increase in insulin resistance and prevents glucose entry into the cell, all mechanisms designed to prevent the adipocyte from accumulating substrates for energy storage. The role of TNF-α in obesity and insulin resistance remains poorly explained. TNF-α may well play a role in the inflammatory response to insulin resistance but secretion from other sources other than adipose tissue may be important.

Leptin from adipose tissue is responsible for an increase in fatty acid (FA) oxidation and reduced esterification in resting rodent skeletal muscle (127). In the absence of leptin, obese human skeletal muscle demonstrates significantly elevated levels of

total FA uptake and enhanced rates of FA esterification into triacylglycerol compared with lean subjects (127). In the presence of leptin, lean muscle demonstrates elevated rates of endogenous and exogenous palmitate oxidation. Leptin also reduces the ratio of esterification to exogenous oxidation, demonstrating the increased partitioning of FA toward oxidation and away from storage. Contrary to these findings in lean muscle, leptin has no effect on FA metabolism in skeletal muscle of the obese. Thus, leptin increases FA oxidation in skeletal muscle of lean, but not obese humans, demonstrating the development of leptin resistance in obese human skeletal muscle (127). Leptin has a fundamental role to play in adipose tissue regulation both in health and disease, some of which influences skeletal muscle.

Adiponectin induces fatty acid oxidation in skeletal muscle and reduced levels are associated with insulin resistance both in mice and humans (152, 153). The proposed mechanism centres around activation of AMP activated protein kinase (AMPK), which is known to regulate cellular malonyl CoA concentrations by inhibiting acetyl CoA carboxylase (154). Consequently there is a decrease in lipogenesis associated with increased mitochondrial fatty acid beta-oxidation. Adiponectin may also have a role in damping down the inflammatory effects of TNF-α, while TNF-α and IL-6 have a role in decreasing adiponectin levels (155) (Figure 1.4).

Adipokines and myokines interact and play a complex role in insulin resistance, obesity, and the regulation of skeletal muscle. It would appear that regulation of any of these adipose – muscle derived cytokines has a direct effect on the others and the mass / composition of a variety of tissues in the body. Adipokines have a number of primary and secondary effects throughout the body; however, all seem to be associated directly or indirectly in regulating a chronic inflammatory state which influences insulin resistance and body composition. Adipokines may also have a role in pre-cachexia, cachexia and refractory cachexia states. Disruption in the normal physiology of fat metabolism in cancer plays a crucial role in distant organ signalling. On development of insulin resistance, other organs such as the liver, skeletal muscle, and the hypothalamus alter their responsiveness and change how they react to insulin (109). Therefore adipose tissue and skeletal muscle can be seen as a producer of cytokines, hormones, and lipids that signal these distant organs to regulate systemic metabolic homeostasis and metabolism. Therapeutic targets may be available to modulate this process. There is little doubt that adipokines and myokines are

intricately involved in adipose – muscle tissue cross talk both in normal physiology and pathophysiology.

Factors such as zinc-α2-glycoprotein (ZAG, or otherwise known as lipid mobilizing factor) (156), known to degrade adipose tissue and proteolysis-inducing factor (PIF) (157) known to degrade muscle have been linked with weight loss in cancer patients (158), although the human form and mechanism of PIF in cancer cachexia has yet to be fully identified (159).

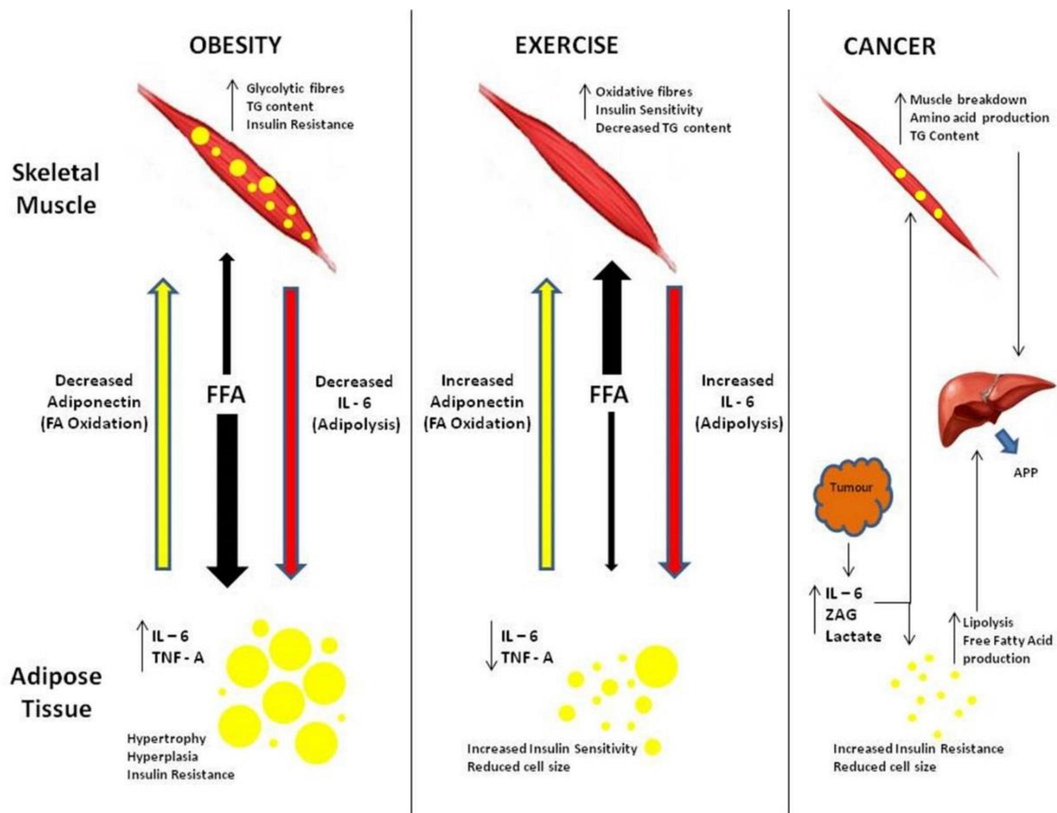


Figure 1.4 - Adipose - muscle cross-talk. The main myokines and adipokines involved in the potential interactions are shown. Obesity and exercise represent opposite situations concerning expression of these molecules and thus different metabolic balance concerning fatty acid metabolism and insulin sensitivity. In cancer patients the secretion of the main cytokines leads to degradation of both adipose and muscle mass. FA, Fatty acid; FFA, free fatty acid; TG, triglycerides. (Figure adapted (160))

1.7 Fat loss

The incidence of obesity and diabetes continues to increase worldwide. The obese patient is generating a new challenge in medicine and treatment. The physiology of obese patients differs remarkably from a normal weighted individual. Recently, patients suffering from advanced cancer have been found to be overweight rather than underweight (161). This has been shown to confound conventional measurements for risk stratification such as BMI. A recent study of pancreatic cancer patients has shown that severe muscle depletion when combined with obesity to be an independent adverse prognostic indicator in this patient group and should be considered as an alternative and more powerful means of risk stratification (12). It is, however, unclear how muscle depletion combined with overweight / obesity causes accelerated demise. The adipokines secreted by excess adipose tissue may act as systemic inflammatory mediators, inducing of insulin resistance in skeletal muscle and leading to a further increase in muscle protein loss. Increased lipolysis appears to be a key factor underlying fat loss, though decreases in lipid deposition and adipocyte development may also contribute (162). The balance between lipogenesis and lipolysis is integral to the net loss of adipose tissue in cancer cachexia and genes regulating this process may vary in different individuals, this is discussed further in Chapters 5 and 6.

1.8 Muscle loss

In healthy adults, skeletal muscle mass is maintained within relatively narrow limits, reflecting a dynamic balance between protein synthesis and degradation. A predominance of either will result in muscle hypertrophy or atrophy. Even small changes in protein synthesis or degradation will lead to large protein deficits over time due to the continuous process of protein turnover. In cancer cachexia, there is ongoing debate as to whether a reduction in protein synthesis, an increase in protein degradation or a combination of both is more relevant. Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients' function and quality of life and is clearly associated with a poor outcome (11, 12, 69). The balance between synthesis and degradation is

integral to the net loss of muscle tissue in cancer cachexia and genes regulating this process may vary in different individuals. The known morphology, mediators, and mechanisms involved in controlling muscle mass in cancer cachexia are discussed in detail throughout this thesis (Chapter 2) and for the first time a validated definition incorporating muscle mass (Chapter 4) is used in the discovery of SNPs in genes regulating these processes (Chapter 6).

1.9 Biomarkers

Refractory cancer cachexia is easy to identify, however, at such an advanced stage it is usually a pre terminal event and very difficult to treat. Early identification of patients at risk of cachexia and the institution of prophylactic measures to attenuate its progression is vital to patient survival and outcome. Biomarkers which are readily assessable in patients with cachexia need to be identified (Figure 1.5). Being able to predict who is at risk from developing cancer cachexia will help in diagnosis and provide a platform to assess novel therapies aimed at treating cancer cachexia.

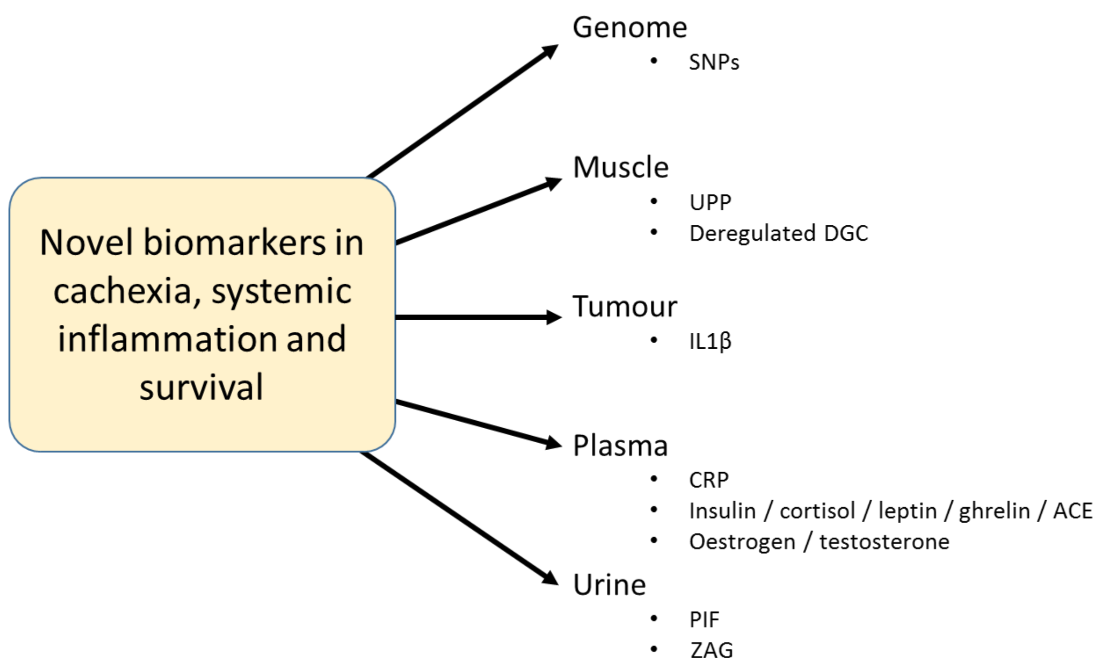


Figure 1.5 - Potential sources of available biomarker development in cancer cachexia

1.9.1 Plasma biomarkers of cachexia

As mentioned previously, CRP is a robust marker of systemic inflammation in cancer patients and has been associated with the presence of anorexia, hypermetabolism, accelerated weight loss and shortened survival (163-165). The acute phase response itself has been implicated in the pathogenesis of cancer cachexia (22). The presence or absence of an APR as indexed by an elevated CRP has been incorporated into a three-factor mathematical definition of cachexia for patients with pancreatic cancer (22). Whilst the underlying process of inflammation seems to be a key factor in the progression of cancer cachexia, other cytokines have been studied as potential biomarkers (166-169). The latter however, have not shown the reproducibility of CRP, this may be due to the complex interplay of local versus systemic mediators of inflammation and the differing processes they initiate on varying tissues (as described in section 1.7.1). In relation to the neuroendocrine axis affecting food intake, insulin resistance and hypercortisolaemia have been documented in the cachectic cancer patient (170, 171). However, these have not been studied in detail in terms of the early cachectic process. Angiotensin II produces muscle catabolism and weight loss in murine models and such effects have been attenuated by angiotensin converting enzyme (ACE) inhibitors (172), this may represent a future biomarker for cancer cachexia. Lastly, leptin and ghrelin are implicated in the regulation of food intake (173, 174). All these potential mediators represent targets for development of novel biomarkers to assess patients at risk from developing cancer cachexia.

1.9.2 Muscle biomarkers of cachexia

The main details of muscle biology in cancer cachexia are discussed in Chapter 2. Briefly, the molecular mechanisms surrounding muscle atrophy (UPP and autophagy pathway) or hypertrophy (mainly IGF-1 mediated signalling) have now been described in detail (18). Cancer cachexia leads to a complex alteration in these mechanisms and is likely to cause suppression of the hypertrophy pathways and upregulation of the atrophy pathways (175). The ubiquitin–proteasome pathway (UPP) is a catabolic pathway in cancer cachexia and in acute animal models seems to be the main pathway attributed to excess skeletal muscle wasting (82). The UPP has been documented in rodent models of cancer-associated muscle-wasting to be overactivated (81). Although this has been carried through to human research, it has

not been a consistent finding (176, 177). It is thought that the presence of systemic inflammation may lead to the over-activation of the UPP (178). Recent data have suggested that systemic inflammation may act in part through dysregulation of the dystrophin glycoprotein complex (DGC) (179). The DGC is a muscle-specific protein manifold that anchors muscle fibre membranes in place and prevents them from being torn by shear forces produced during muscle contraction. The dysregulation of DGC has been shown to correlate positively with weight loss in patients with gastrooesophageal adenocarcinoma (179). It remains, however, difficult to use skeletal muscle as an early biomarker as, at present, this would require percutaneous or open biopsy.

1.9.3 Urinary markers of cancer cachexia

Urine is also useful to both observe and to screen for potential biomarkers as it can be sampled very easily in a non-invasive way. Urine can also be collected in large quantities, is comparatively stable in terms of its molecular constituents, and has been shown to yield both metabolic (180, 181) and proteinaceous (182-184) discriminators in patients with pancreatic cancer. These 'fingerprinting' approaches have the advantage of predictive models with higher accuracy than single markers alone but their applicability in the clinical setting is still questionable due to technical issues. Multiplexing of various specific markers, e.g. the reported 810 antibody-panel (185), might provide a way forward, though economically this might be not feasible as a general screening tool. Single molecule identification of urinary markers in pancreatic cancer using mass spectrometry has yielded a number of interesting potential biomarkers (e.g. annexin A2, gelsolin, CD59 and S100A9) (182), though the utility of these markers is unknown due to a lack of further validation. However, independent studies have shown that some proteins such as S100A9 (186), CD59 (187) and gelsolin (188) are at least associated with cancer of the upper gastrointestinal tract or cultured pancreatic cells.

Proteolysis-inducing factor was first identified as a glycosylated polypeptide using an antibody cloned from splenocytes of mice transplanted with the murine adenocarcinoma 16 (MAC16) tumour (189). This antibody bound to a 24kDa sulphated glycoprotein, which was also present in the urine of cachectic human cancer patients (189). A hybridoma was subsequently developed for the production of the antibody, for use in purification of PIF by affinity chromatography and for assay

of physiological samples. Immunoreactivity with the murine anti-PIF antibody has since been described in the urine of weight-losing patients with carcinoma of the pancreas, breast, ovary, lung, colon, rectum and liver (189). Patients with pancreatic cancer demonstrating PIF immunoreactivity in urine had a significantly greater total weight loss and rate of weight loss than patients whose urine did not (158). Furthermore, isolation of protein with PIF immunoreactivity from human urine induced muscle wasting when injected into mice (190). PIF is thought to induce muscle wasting via both increased degradation and decreased synthesis of muscle protein (191).

Urine in particular has the potential to yield many more specific biomarkers due to its complexity to contain far more observable proteins than previously thought (LSSR database at www.PADB.org lists more than 5000 unique proteins), and identification of valid biomarkers and devising specific clinically-transferable detection methods might allow large-scale preventative/diagnostic as well as prognostic disease screening in the near future.

1.9.4 Genetic biomarkers of cachexia

Currently with all the available patient demographics and clinical risk factors for cachexia, it is not possible to predict who will develop cancer cachexia and who will not (Figure 1.6). Ultimately, with the advent of new biomarkers this may be possible, but even more important is the identification of the speed at which a patient develops the syndrome. It has been proposed such a variation may, in part, be due to the patient's genotype rather than the tumour phenotype (Figure 1.6).

Genetic association studies are one of the most common approaches to find genes involved in complex traits. The candidate gene approach to conducting genetic association studies focuses on associations between genetic variation within pre-specified genes of interest and phenotypes or disease states. Association studies identify polymorphisms in which an allele occurring in the general population occurs at a different frequency in the disease group. In these instances, the disease associated allele does not cause the disease in the same way that a Mendelian mutation does, but increases susceptibility to the disease as a genetic risk factor, most likely in conjunction with other genetic and/or environmental risk factors.

Association studies can either be direct or indirect. In direct association studies, target polymorphisms which are themselves putative functional variants (for example a SNP variant in a gene at a codon that changes an amino acid) are genotyped in both the general (control) and also trait (disease) population. A statistically different frequency of the alleles and/or genotypes in the control population versus the disease group would suggest that the polymorphism in question has a direct effect on disease pathogenesis. However, it is likely that many causal variants contributing to complex disorders will be non-coding. These variants could include those that affect gene regulation, expression or alternative splicing and such functional variants are difficult to predict. For this reason, most association studies are indirect; where the polymorphisms genotyped in the control populations and trait populations are surrogates for the unknown causal locus.

Identifying susceptibility genes for complex disorders by the indirect method depends on the existence of an association between the causal variants and surrounding polymorphisms nearby. This association is termed linkage disequilibrium (LD) and is defined as the non-random association of alleles at two or more loci and describes a situation in which correlation between nearby variants such that the alleles at neighbouring markers (observed on the same chromosome) are associated within a population more than if they were expected by chance. Candidate genes are most often selected for study based on a prior knowledge of the gene's biological functional impact on the trait or disease in question. The rationale behind focusing on allelic variation in specific, biologically relevant regions of the genome is that certain mutations will directly impact the function of the gene in question, and lead to the phenotype or disease state being investigated.

Suitable candidate genes are generally selected based on known biological, physiological, or functional relevance to the disease in question. This approach is limited by its reliance on existing knowledge about known or theoretical biology of disease. However, more recently developed molecular tools are allowing insight into disease mechanisms and pinpointing potential regions of interest in the genome. Genome-wide association studies and quantitative trait locus (QTL) mapping examine common variation across the entire genome, and as such can detect a new region of interest that is in or near a potential candidate gene. Microarray data allow researchers to examine differential gene expression between cases and controls, and can help pinpoint new potential genes of interest. In addition, the availability of genetic

information through online databases enables researchers to mine existing data and web-based resources for new candidate gene targets. Cancer cachexia is a multifactorial disease caused by the interplay of a number of different processes all driven by different genes which would make it highly suited to a candidate gene approach. However to identify a genetic predisposition or biomarker to cancer cachexia, an exhaustive review of known functional or influential polymorphisms involved in the causative processes is needed to conduct a candidate gene study.

1.9.5 Candidate gene association analysis in cachexia

The finding that only a proportion of patients with chronic disease develop cachexia has prompted studies looking for genetic polymorphisms that may underlie differential susceptibility (192) (Figure 1.6). The most frequent targets for these studies to date have been genes encoding pro-inflammatory cytokines, such as TNF-α and some of the interleukins. This is based on the hypothesis that continued systemic inflammation plays a central role to the pathogenesis of cachexia. However, as new pathways and genes evolve (specifically in muscle and adipose tissue biology) and phenotypic definitions continue to evolve, there is a need to re-strategise the selection of candidate genes to drive association studies.

The case to support a genetic predisposition to cachexia is strengthened from the known genetic contribution to the activity of a variety of key mechanisms that underlie the cachexia syndrome. In a previous systematic review on the identification of possible genetic polymorphisms involved in cancer cachexia, a total of 184 polymorphisms with functional or clinical relevance to cancer cachexia were identified in 92 candidate genes (193). Following this review, a candidate gene study identified 23 significant SNPs associated with cachexia based on definitions of weight loss and systemic inflammation (as indexed with CRP), and a SNP in the SELP gene encoding for P – Selectin was validated in an independent cohort. P – selectin binds to leukocytes and in certain inflammatory conditions, the plasma concentration of soluble P – selectin is elevated (194). Most of the work on identification of SNPs involved in cancer cachexia to date has led to the discovery of other SNPs involved in the innate immune system, mainly in the interleukin family of cytokines (195-202) (Table 1.2). SNPs in other biological processes have also been studied for their association with cancer cachexia (203, 204) (205) (Table 1.2). By altering the

phenotype to include a degree of skeletal muscle quantification, it may be possible to identify SNPs associated with muscle tissue biology as altered in cancer cachexia.

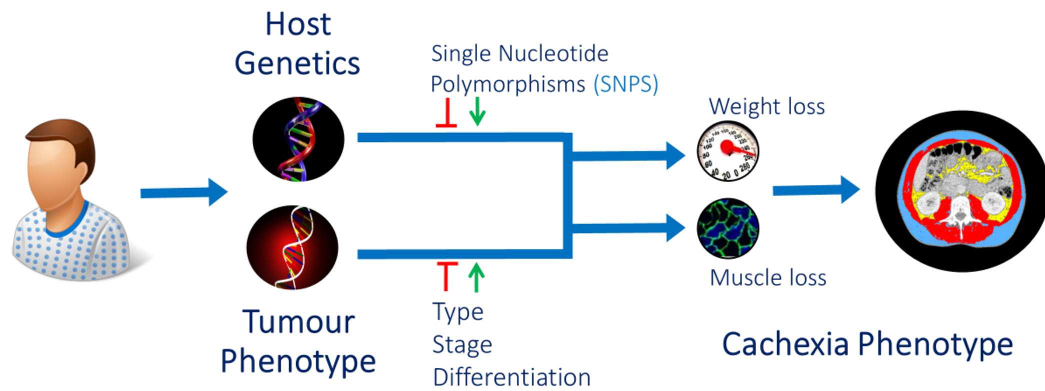


Figure 1.6 - In the post human genome era, one of the major goals of biology is to bridge successfully the gap between genotype and phenotype. This process of linking genotype and phenotype plays a crucial role in understanding the numerous and varied biological processes which contribute to overall cellular, tissue and organism responses, particularly within a disease state such as cancer cachexia. By identifying SNPs in the patients genotype it may be possible characterise any potential relationship between patients' genotype and propensity to develop cancer cachexia.

Author	SNP	Gene	Patients included	Phenotype
Avan (206)	rs6136 rs1130233	SELP AKT1	Test and validation cohorts of 151 and 152 chemo-naive locally-advanced/metastatic PDAC patients	Unintended weight loss of more than 5% of body weight or weight loss of more than 2% in already depleted individuals with a body mass index (BMI) less than 20 kg/m2 over 6 months
Solheim (204)	No associations	N/A	1853 patients with cancer at different sites, stages and with different performance status	EORTC QLQ-C30 questionnaire, question 13: “have you lacked appetite”.
Punzi (205)	rs1544410 (BsmI) rs731236 (TaqI)	VDR	43 patients with cancer from various sites and stages	Guidelines for diagnosis of cancer associated cachexia provided by the Italian Association of Medical Oncology
Tan (207)	rs6136	SELP	775 patients with UGI and pancreatic cancers	6 Phenotypes (1) >5% Weight loss (2) >10% Weight loss (3) >15% Weight loss (4 – 6) The above with CRP concentration of >10 mg/l $^{-1}$
Solheim (203)	No associations	N/A	1797 patients with cancer at different sites, stages and with different performance status	• BMI: <20 kgm^{-2} • Karnofsky score: <80 • CRP: <10 mg l^{-1} • Appetite loss: a response of little or greater on EORTC QLQ-C30 item ‘have you lacked appetite?’ >3 features = Severe cachexia 2 or 3 features = Mild cachexia <2 = No cachexia
Sun (208)	rs1800896	IL-10	223 Gastric cancer	Not clear
Bo (196)	rs2227306	IL-8 +781	Gastric cancer	Not clear
Jatoi (198)	rs1800629	TNF	471 patients with non-small cell lung cancer	>10% Weight loss
Rausch (199)	rs3024498	IL-10	1149 Caucasian lung cancer survivors	Lung Cancer Symptom Scale How much are you experiencing loss of appetite?
Deans (200)	rs1800896	IL-10 (-1082)	203 patients with upper gastrointestinal (UGI) cancers	>10% Weight loss
Knoll (201)	rs2229616 Val103Ile	MC4R	509 patients with various cancers (including	(1) >10% Weight loss (Exclusivley cancer)

			haematological malignancies) at various stages	(2) >10% Weight loss (Treatment influenced) (3) >5% Weight loss (4) >1% Weight loss (Cancer specific) or >5% treatment induced (5) No weight loss
Zhang (209)	rs1143634	IL-1β (+3954)	214 Patients with locally advanced gastric cancer	>10% Weight loss
Jatoi (197)	rs1143634	IL-1β (+3954)	44 Patients with metastatic gastric and gastro-oesophagal cancer	Phenotype is greater improvements in weight registered every 3 week during chemotherapy

Table 1.2 - Published associations of genetic variants in cancer cachexia

1.10 Clinical Consequences and Management of Cachexia

1.10.1 Reduced physical performance

The objective clinical consequences of cachexia are profound including shortened survival (23), impaired response to anti-cancer therapy (23), impaired immunity (210), reduced performance status (PS) (211), reduced physical activity (PA) (211) and worsened QoL (Figure 1.7). Depletion of skeletal muscle is the key component of weight loss that underpins many of the symptoms experienced by cachectic cancer patients. Muscle wasting leads to reduced exercise capacity and increased fatigue (212), which results in the lower PS scores, reduced PA and worsened QoL scores observed. It has been proposed that the response to muscle loss and function in cancer cachexia may be sexually diamorphic (213). In gastrointestinal cancer patients, it has been shown that there is variability in lower limb muscle function, mechanical quality and mass according to both the degree of weight loss and patient sex (213). While lower limb muscle mass, strength and power was shown to decline 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. These changes

lead to a significant reduction in patient’s quality of life. One proposed mechanism to account for sexual dimorphism may be to do with sex hormone production. It has been demonstrated that hypogonadism (low total and/or free testosterone levels) is associated with poorer survival in male cancer patients (214). It is also well established that low testosterone levels impact on muscle mass and function (214). Furthermore, testosterone replacement has been shown to prevent loss of muscle in ageing males (215). These findings are consistent with recent literature suggesting sexual dimorphism may impact on the effects of systemic disease such as cirrhosis (216) and must be considered when treating cachexia in clinical practice. Cachexia has a detrimental effect on a patient’s QoL. Patients with cancer cachexia report altered body image, which impacts their emotions, spirituality, relationships, and social functioning. Lives are restricted and isolated, which is compounded by emotional distancing by carers and healthcare professionals (217). These patients also experience anorexia and increased fatigue (22, 218). Overall, this results in decreased performance status and QoL indices (219).

The devastating effect of cachexia is further characterized by considering the free-living physical activity of patients. In a study examining patients using an electrical activity monitor worn over a period of 1 week, patients with cancer cachexia demonstrate a 40% reduction in the level of physical activity (211).

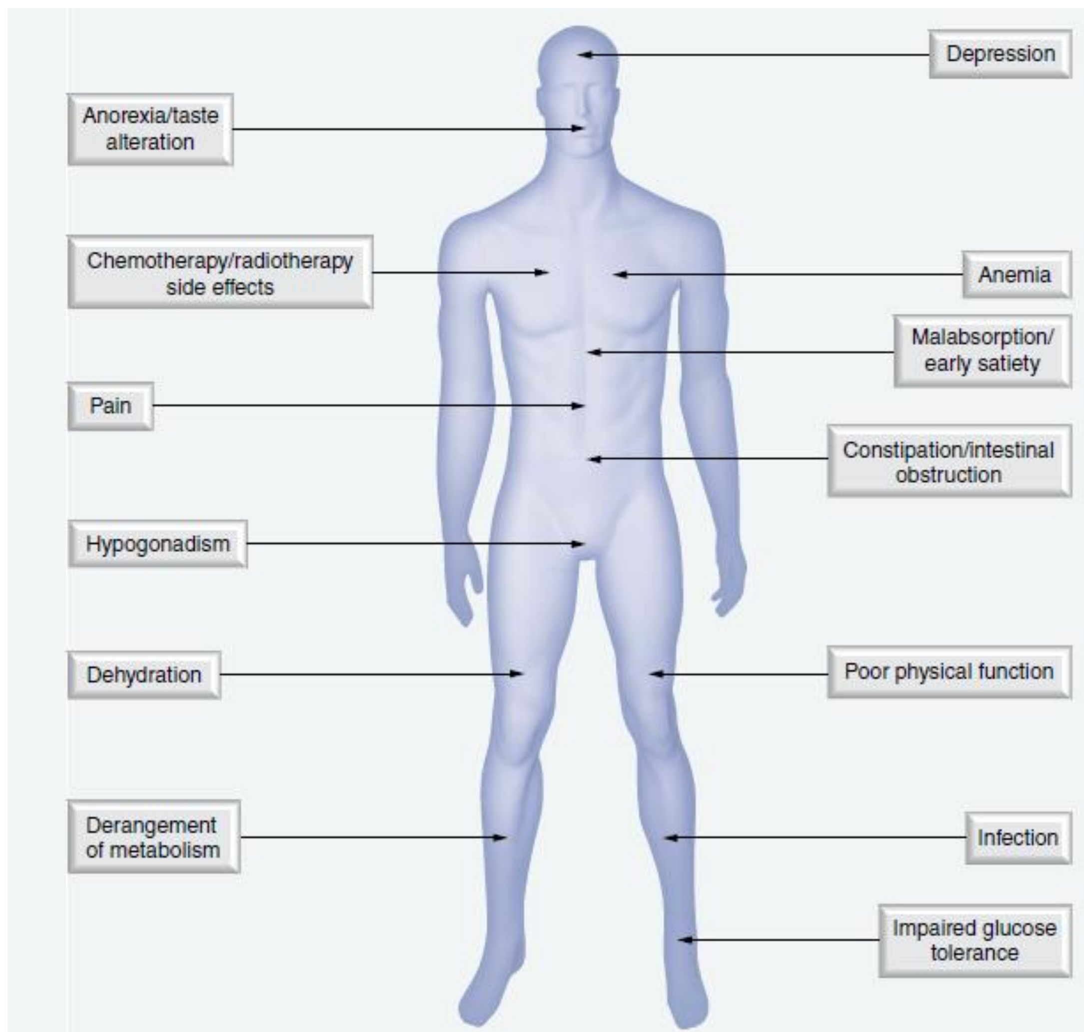


Figure 1.7 – Clinical features frequently associated with cancer cachexia

Many other patient variables contribute to the heterogeneity of cancer cachexia such as age, co morbidity, stage of disease, treatment, and genetics. An individualised management plan can be based on the patient's baseline characteristics and the mechanisms most likely to contribute to weight loss and their potential reversibility. Many cancer patients undergoing treatment are malnourished and a number of interventions such as chemotherapy and radiotherapy further exacerbate this. A multi-professional and multimodal approach, including nutrition and physical activity interventions aimed at preventing or delaying cancer-related malnutrition has been suggested to be of key importance for the efficacy of the oncological therapies (220). A number of RCTs have also demonstrated adequate feeding via enteral or parenteral methods improve outcome and reduce the side effect profile of chemo / radiotherapy

and surgery (221, 222). Prevention of the anorexia component of cancer cachexia via adequate nutrition remains a primary goal in the treatment of cancer, but other mechanisms are at play that prevent primary nutrition reversing the weight loss. Exercise may help prevent anabolic resistance observed in older adults or in immobilised or chronically ill patients by encouraging muscle protein synthesis and anabolism. Proposed mechanisms whereby exercise may have these effects include improved amino acid sensitivity and (or) insulin sensitivity of muscle(223). Recent research has also demonstrated that immobilisation induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion (224). Post-absorptive muscle protein synthesis is reduced in the muscle of an immobilised limb. It has been shown that suppressed muscle protein synthesis also extends to the postprandial state, even when the free amino acid concentration is high.

1.11 Hypotheses

1. Regardless of tumour type, host genetic variation contributes to the prevalence of cancer cachexia
2. New biological mechanisms, new genes, and new signalling pathways are being discovered that may influence the course of cancer cachexia in general and cancer associated muscle atrophy in particular
3. Genetic variants that associate with cancer cachexia may differ according to the phenotypic definition of cancer cachexia (especially in the presence or absence of muscle fibre atrophy)
4. Skeletal muscle atrophy may not be clearly identified by the presence of weight loss and may be better identified by a combination of weight loss and low muscularity (identified on diagnostic CT scan)
5. Gene expression in muscle may vary with pro-cachectic genotypes

1.12 Aims

1. To refine clinical identification of cancer patients with muscle fibre atrophy using a combination of self-reported weight loss and low muscularity (identified on diagnostic CT scan) as defined as per international consensus definition of cancer cachexia
2. To undertake a systematic review to explore new genetic polymorphisms in potential candidate genes involved in the development of cancer cachexia
3. To validate previous findings of genes associated with cancer cachexia as defined by weight loss alone
4. To undertake genetic association studies assessing for susceptibility to cachexia as informed by the results of the systematic review and the presence of muscle fibre atrophy as defined by a combination of weight loss and low muscularity
5. To look for evidence from the transcriptome in skeletal muscle of genes identified in the genetic association studies being altered at the transcriptional level

1.13 Plan

Weight loss has long been seen as the hallmark of cancer cachexia and the relationship between changes in total body weight over time and cancer outcomes are highly correlated. It is widely accepted that changes in body weight may involve any of its major tissue compartments (lean tissues, adipose tissues, and bone) and that within these compartments; there may be specific changes in the mass of individual organs and tissues.

In Chapter 1, the rationale and background to a potential genetic predisposition to cancer cachexia is reviewed. Many of the current associations have been found exclusively in the genes regulating inflammation, however by incorporating skeletal muscle into the phenotype definition of cachexia and studying SNPs associated with altered muscle mass / function it may be possible to identify new targets by using an updated candidate gene list.

With skeletal muscle being shown to be an increasingly important marker of outcome and survival and also being incorporated into a definition of cancer cachexia (11), Chapter 2 outlines the current knowledge of the mechanisms associated with changes in skeletal muscle composition from animal models and how these have been related to human models.

Chapter 3 outlines the materials and methods involved in all the studies in the thesis.

Chapter 4 investigates the changes in muscle fibre biology with regards to morphological structure and composition, the alterations in various pathways that may account for altered fibre size and relates these changes to the different clinical diagnostic criteria that have been proposed.

Chapter 5 presents a contemporary review of the literature with regards to a greater understanding of the processes involved in cancer cachexia. To enable further candidate gene selection studies, new targets need to be identified in order to maximise the potential for associations. To accommodate the evolving phenotype definitions and the current state of the understanding of cachexia, candidate genes or pathways related to the biology of muscle, inflammation, adipose tissue, obesity, diabetes and molecular mechanisms of cancer in general, as well as factors affecting survival and prediction of outcomes following treatments were researched for relevant SNPs.

Chapter 6 utilised a candidate gene approach to evaluate the association between genetic polymorphisms and the presence of cancer cachexia in patients recruited across six centres. The primary objective of this study was to further replicate our findings from a previous association study which identified the SNP rs6136 in the SELP gene and a number of other polymorphisms as promising candidates influencing genetic predisposition to cachexia. The second objective was to explore the association with 92 predefined new candidate SNPs and WL. The third objective was to compare the entire panel of candidate SNPs and their association with LM with and without WL. Also investigated was whether genes demonstrating significant associations with the cachexia phenotypes had altered transcript expression in muscle from cancer patients with or without those phenotypes.

Chapter 7 summarises and discusses the relevance of the work presented throughout the thesis. It also outlines the significance of the findings in relation to current work and where the work may be taken in the future.

CHAPTER 2

Mechanisms and Mediators of Muscle Wasting in Animal Models and in Humans with Cancer Cachexia

2.1 Summary

Skeletal muscle loss appears to be the most significant clinical event in cancer cachexia. When considering how to phenotype cachexia for genetic association studies, muscle wasting is therefore a key domain. With regards to such muscle loss, despite extensive study in a range of models, there is ongoing debate as to whether a reduction in protein synthesis, an increase in degradation or a combination of both is the more relevant. Each model differs in terms of key mediators and the pathways activated in skeletal muscle. Certain models do suggest that decreased synthesis accompanied by enhanced protein degradation via the ubiquitin proteasome pathway (UPP) is important. Murine models tend to involve rapid development of cachexia and may represent more acute muscle atrophy rather than the chronic wasting observed in humans. There is a paucity of human data both at a basic descriptive level and at a molecular / mechanism level. Progress in treating the human form of cancer cachexia can only move forwards through carefully designed large randomised controlled clinical trials of specific therapies with validated biomarkers of relevance to underlying mechanisms.

2.2 Introduction

Weight loss in cancer patients is associated with excess morbidity and mortality (4, 5). Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life and duration of survival. Cancer cachexia has recently been defined 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 (11). Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients' function and quality of life and is clearly associated with a poor outcome (11, 12, 69).

2.3 Heterogeneity

Heterogeneity both in clinical and animal models is one of the key issues that has impaired research into cancer cachexia. Cachexia is not a single phenomenon but evolves through a spectrum of pre cachexia, cachexia, and refractory cachexia (11). The incidence and severity of cachexia can vary according to tumour type, site, and mass (23) as well as the individual host response. Equally, the contribution of reduced food intake versus abnormal metabolism can vary considerably (225). The components of such abnormal metabolism also vary, and within the same model / individual can evolve with time such that hypermetabolism (226) or activation of proteolytic pathways (227) occurs during the early phase of cachexia but not during a more advanced phase. In humans the cause of such heterogeneity relates not only to the clinical status of the patient and specific effects of the tumour (e.g. causing bowel obstruction, tissue destruction or concomitant infection) but also to co-existing morbidities (e.g. heart failure, chronic renal failure, or chronic obstructive pulmonary disease), age-related sarcopenia and the possibility of a genetic predisposition to develop cachexia (Figure 2.1).

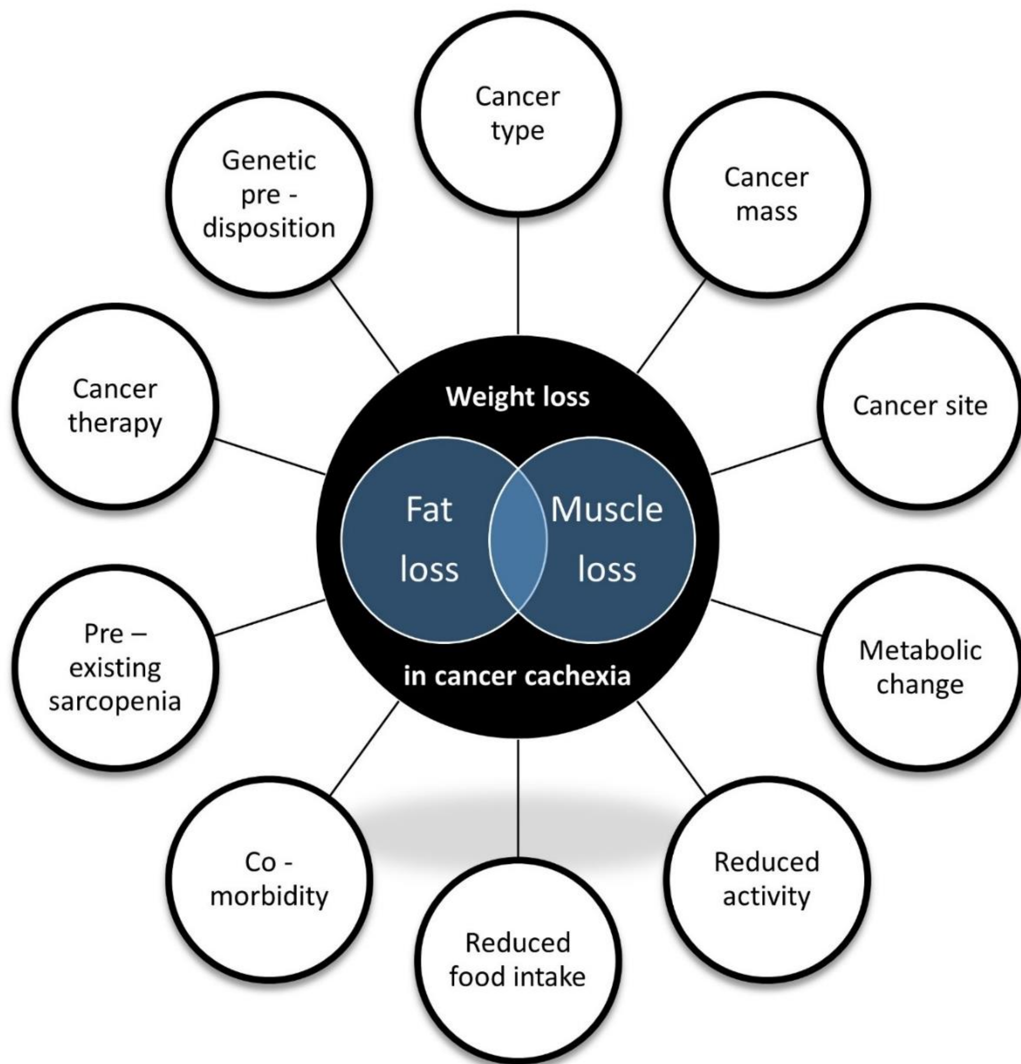


Figure 2.1 – Heterogeneity in cancer cachexia. Weight loss is the key diagnostic criteria for the presence of cancer cachexia. In turn, weight loss may be due to loss of fat and / or lean tissue. Skeletal muscle is a major component of lean body mass and represents the main labile protein reserve in the body. Many factors influence the extent to which fat or skeletal muscle is lost in cancer patients.

There are numerous animal models of cancer cachexia. A recent review concluded that for research into cancer cachexia where there is little evidence of systemic inflammation, the murine adenocarcinoma 16 mouse (MAC-16) and XK1 murine tumour models are useful (228). Weight loss in mice bearing the MAC-16 tumour appears to be independent of reduced food intake or inflammation (228). In contrast the XK1 model demonstrates reduced food intake. All other models induce a host inflammatory response. In the Walker 256 and MCG - 101 models tumour growth is

extremely rapid resulting in a tumour mass exceeding 10% of host body weight in a matter of days (228). This highlights the problems with the translational value of relatively acute onset cancer cachexia seen in murine models compared with humans where a cancer may spread over a period of months or years (with similar time scale for the evolution of cachexia). One of the most popular models at present is a particular clone of the colon-26 adenocarcinoma (C26) tumour in mice. This is thought to be a mainly IL-6 dependant model of cachexia (107). Whether, in fact, this can be considered as representative of the majority of cancer patients remains highly speculative.

2.4 Skeletal muscle morphology in cancer-associated myopenia: murine models

Skeletal muscle is composed of muscle fibres which are classified according to their speed of contraction and predominant type of energy metabolism. Muscle fibres can be classified as type I, slow-twitch and type II, fast-twitch fibres based on their predominant myosin heavy chain (MyHC) isoform content. Generally, type I and type IIa fibres utilise oxidative phosphorylation as their energy source, whereas type IIx and IIb fibers harness anaerobic metabolism to generate ATP (229, 230). Both the percentage and structural morphology of the fibre type will determine the phenotypic capacity and functional performance of any given muscle. Environmental factors in both health and disease have a direct impact on muscles leading to changes in fibre type and morphology which lead to changes in muscle functionality; such processes include aging, exercise, diabetes, disuse atrophy, chronic heart failure, and cachexia (231-244). The change, preservation or loss of fibres may influence clinical symptoms and there is some evidence that fibre type is targeted selectively in cancer cachexia (70, 71). Ongoing loss of protein in muscle tissue leads to muscle fibre shrinkage and a reduction in CSA. This loss of CSA leads to the main muscle groups in the body undergoing changes in fibre composition which leads to a marked loss of aerobic capacity (VO^2max) in healthy subjects as well as cancer patients (237, 245-248).

2.4.1 Fibre size and type in cancer associated myopenia

The model that has been most frequently studied is the C26 tumour grown subcutaneously in mice (Table 2.1). The predominant muscles studied include the gastrocnemius and soleus from the lower limb. Unlike humans, no model uses old, co-morbid mice undergoing concomitant anticancer therapy! A common finding is a decrease in fibre size. With regards to fibre type, evidence from animal models suggests that type II fibres are targeted selectively (179) with relative preservation of type I fibres in fasting (249), exposure to Glucocorticoids (250, 251), sepsis (252) and in the gastrocnemius muscle of the C26 model of cancer cachexia (253). Not all studies have demonstrated type 1 and 2 fibre differences. Indeed, in a recent exposition of the C26 cachectic mouse model, both glycolytic and oxidative fibres of (extensor digitorum longus) EDL muscle underwent wasting (254), whilst in a previous study using the same mouse model there was a significant increase in the amount of type 2b MyHC and a significant decrease in the amount of type 1 MyHC in soleus muscle (255).

The activity patterns of muscle are also key in determining phenotype. If muscle cells are recruited infrequently, they develop into fast/glycolytic units whereas if they are recruited more often, they form slow/oxidative units. In the C26 mouse model of cancer cachexia, there have been reports of switching of myosin isoforms in the soleus muscle of cachectic mice (255).

Authors	Model	Muscle group	Myosin Fibre Type	Fibre Size (FCSA) (μm^2)	Fibre Percentage (%)
Aulino (254)	Mouse C26 Colon	EDL	1 2	↓ ↓	↓ ↓
Acharyya (179)	Mouse C26 Colon	TA / Gastrocnemius	1 2	**No change** ↓	**No change** ↓
Diffee (255)	Mouse C26 Colon	Soleus	1 2a 2b		↓ **No change** ↑

Table 2.1 – Experimental animal data on myosin composition of muscles in cancer cachexia

2.4.2 Fibre RNA, DNA, and protein content in cancer associated myopenia

A reduction in total RNA content has been associated with a depression in protein synthesis in mice bearing the human hypernephroma XK1 (256) and in mice bearing the MAC-16 tumour (257). Timing of sampling may be important in the results obtained from such muscle biopsies. A reduction in the RNA content in the muscle of mice bearing the Ehrlich ascites tumour has also been reported, but this occurred later than the onset of depressed protein synthesis (258).

Protein content expressed in relation to wet weight of muscle has been shown to decrease progressively (in excess of 50%) in the gastrocnemius muscle of mice bearing the MAC-16 tumour (257). Similar changes have been documented in the Yoshida ascites hepatoma A130 cachexia model (259). This suggests that not only is there loss of fibre diameter, but that the quality of the fibre is altered with loss of either sarcoplasmic or myofibrillar protein. DNA content of the gastrocnemius muscle has been shown to remain relatively constant, despite the finding of a decrease in protein

and RNA content (257). Clearly not all DNA in a muscle can be ascribed to myonuclei. However, these findings are consistent with the trend towards fibre cytoplasmic atrophy without a decrease in myonuclear number observed in mature mice that undergo long term denervation injury (260). The issue of whether nuclear domain size is reduced in cancer cachexia remains to be resolved. In particular, whether apoptosis in skeletal muscle is increased in cancer cachexia (261) and the degree to which DNA content is maintained or not via a compensatory increase in myonuclear number (possibly via satellite cell nuclei incorporation) is not known. Features of cachexia such as hypogonadism (resulting in low testosterone) or systemic inflammation (associated with high IL-6) could influence such regenerative capacity.

2.4.3 Dystrophin glycoprotein complex (DGC)

It is also important to recognise that in cancer cachexia there may be changes in the muscle cytoskeleton as well as in the myofibrillar contractile apparatus. In recent years, it has been demonstrated that alterations in the muscular dystrophy-associated DGC also occur in skeletal muscle (179). Using both the C26 and Lewis lung carcinoma (LLC) murine cancer models, it was shown that muscles from tumour-bearing mice exhibited membrane abnormalities, accompanied by reduced dystrophin (core DGC member) expression and increased glycosylation of DGC proteins (179). The DGC is thought to play a key role in maintaining muscle integrity during contraction cycles and clearly loss of this function may exacerbate overall damage to the muscle.

2.5 Mediators of cancer associated myopenia: murine models

2.5.1 Pro-inflammatory mediators

Mediators regulating skeletal muscle atrophy in cachexia are thought to derive from immune or tumour cells, or the targeted tissues undergoing wasting (both adipose tissue and skeletal muscle). Inflammation is thought to be of paramount importance. Cancer cells rely on the production of pro-inflammatory mediators for growth,

protection from apoptosis, and promotion of angiogenesis/metastasis. The tumour may consequently initiate a cytokine cascade that has multiple, direct, and distant effects including the initiation of skeletal muscle protein degradation.

CHO cells transfected with the human TNF-α gene induced cachexia when implanted into nude mice (262). A similar finding was observed with CHO cells constitutively producing IFN-γ (263). An anti-IFN-γ monoclonal antibody was able to reverse the wasting syndrome associated with the murine Lewis lung carcinoma (264). TNF-α and IFN-γ work co-operatively to down-regulate transcription of the MyHC gene *in vitro* and *in vivo*, but not other core myofibrillar proteins (253). Furthermore, MyHC protein expression was decreased in a specific fashion in the C26 model (253), a largely IL-6 dependent model (265). TNFα has been shown in vitro to inhibit both adipocyte and skeletal myocyte differentiation (113, 114). It also plays a role in insulin resistance attenuating the insulin signalling pathway (115). The presence of TNFα promoted atrophy in cultured myotubes, resulting from the induction of E3 ligase genes that mediate the breakdown of myofibrillar proteins by the UPP (116-119). TWEAK, another important cytokine with structural properties similar to TNFα, can induce a cachectic phenotype in part by the induction of the E3 ligase MuRF1, and the subsequent degradation of MyHC at the thick filament of the sarcomere (266, 267). A variety of tumours have been shown to secrete IL-6 and this can be amplified by host-derived proinflammatory cytokines (e.g., IL-1). Experiments regulating function in tumour bearing mice also support the requirement of IL-6 in cachexia (106, 107, 265). In mice with a mutation in the APC tumour suppressor gene, elevated circulating IL-6 levels are associated with the presence of muscle wasting (268). Knockout of IL-6 prevents loss of muscle weight and epididymal fat, and reduces intestinal polyp number, implying the existence of an IL-6 cytokine amplification loop between host and tumour cells.

Other cytokines may be potential repressors of cachexia. For example, IL-4, IL-10 and IL-13 all demonstrate anti-inflammatory, and hence potentially anti-cachectic activity (269). In the C26 mouse model, IL-10 gene transfer reduced cachexia and prolonged survival (270). Other cytokines (e.g. IL-15) may have potential ‘antioxidant’ properties that can counter the excess levels of reactive oxygen and nitrogen species (caused by the inefficiency of host antioxidant enzymes) that have been proposed as mediators of muscle atrophy (271). IL-15 was capable of inhibiting skeletal muscle wasting in the Yoshida AH-130 rat ascites hepatoma model by decreasing muscle

protein degradation rates (151). Overexpression of IL-15 in cultured myotubes induced a hypertrophic morphology and increased myofibrillar protein accumulation in co-cultured cells (272).

2.5.2 Catabolic mediators

The interplay of anabolic and catabolic hormones has a distinct role in the development of skeletal muscle wasting. The catabolic molecules myostatin and activin are two members of the TGF-β superfamily that play an important role in growth and are thought to be responsible for the development of cachexia in mice (144). In mice, both molecules bind to activin receptor type-2B (ACVR2B), a receptor in muscle, to initiate a signalling cascade leading to increased expression of MuRF1 and MAFbx/Atrogin-1 and subsequent degradation of myofibrillar proteins (79). In mice bearing the C26 tumour, administration of a soluble ACVR2B decoy receptor resulted not only in reversal of muscle wasting but an increase in survival (145).

2.5.3 Anabolic mediators

The anabolic hormone IGF1 has been implicated in the development of cancer cachexia in murine models. In the rat AH-130 hepatoma ascites model of cancer cachexia, muscle expression of IGF-1 mRNA decreased progressively whereas IGF-1 receptor and insulin receptor mRNA levels increased compared with controls (273). Furthermore, circulating levels of IGF-1 and insulin were reduced. However, the exact mechanism of IGF-1 down-regulation and any role in muscle wasting in this scenario is unclear, as administration of exogenous IGF-1 to tumour-bearing rats did not prevent cachexia (273), and other studies have shown, at least in experimental animal models of cancer cachexia, the IGF-1 signal transduction pathway is not down-regulated (274) (275) (276).

2.6 Mechanisms of cancer associated myopenia: murine models

2.6.1 Protein degradation pathways

Skeletal muscle atrophy may occur as a result of decreased synthesis, increased degradation or both (79). In cancer cachexia it is evident that there is a complex interplay between synthesis and degradation. In mice bearing the MAC-16 adenocarcinoma, weight loss was accompanied by loss of whole body nitrogen in proportion to the overall loss of body mass. Using L-[4-3H]phenylalanine to label proteins in the gastrocnemius muscle to determine rates of synthesis and degradation, a significant depression (60%) in protein synthesis occurred in animals with a weight loss between 15 and 30%. This was accompanied by an increase in protein degradation, which increased with increasing weight loss between 15 and 30% (277). Three major pathways that may contribute to such increased protein degradation include the UPP, autophagy / lysosomal pathway, and the calcium dependant enzymes (e.g. calpains) (100).

2.6.1.1 Ubiquitin proteasome pathway

Various factors including malnutrition, impaired physical activity and the production of catabolic molecules (e.g., glucocorticoids and/or cytokines) upregulate rates of muscle proteolysis (278). A common end point in pathways implicated for increased protein breakdown involves two muscle-specific E3 ubiquitin protein ligases, which target specifically a limited number of proteins for breakdown by the Proteasome. These E3 ligases are called MAFbx/Atrogin-1 and MuRF1 (Figure 2.2).

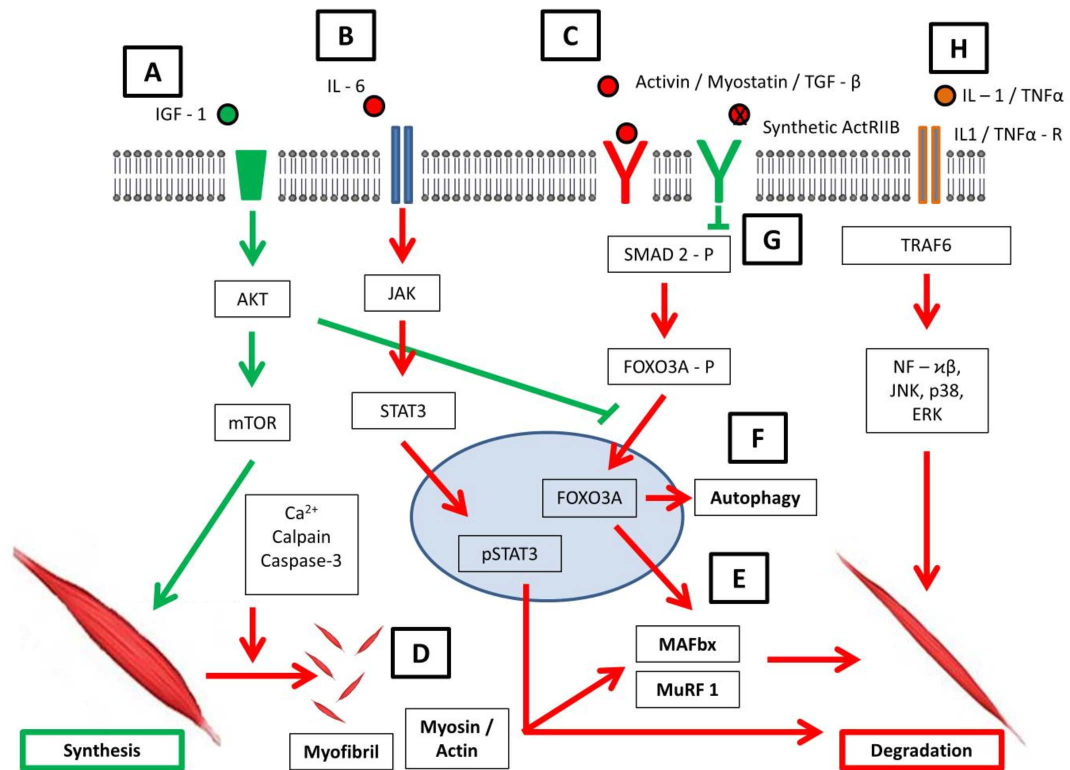


Figure 2.2 - Signalling pathways that regulate protein synthesis / degradation in skeletal muscle. Regulation of muscle synthesis via IGF – 1 induced hypertrophy (A). Myokine activated JAK/STAT pathway involved in the acute phase response and muscle degradation (B). TGF – β induced SMAD pathway, activated by myostatin and activin leading to degradation (C). Calcium mediated mechanisms of muscle breakdown (D). Muscle breakdown via activation of E3 ligases leads to muscle breakdown via the UPP (E). Activation and transcription of autophagy genes (F). Synthetic inhibition of ACVR2B blocks SMAD signalling leading to muscle hypertrophy via blockade of the degradation pathway (G). NF – ϰβ signalling via TRAF6 leading to degradation (H).

2.6.1.1.1 Pro inflammatory cytokine activation of the UPP

TNFα, TWEAK or IL-1 signal to activate the NF–ϰβ pathway. NF–ϰβ influences a variety of pathways including apoptosis, inflammation and differentiation. Pro-inflammatory cytokines induce the cytosolic release of NF–ϰβ from its inhibitory Iϰβ proteins to allow translocation of NF–ϰβ into the nucleus and subsequent transcription of proteolytic pathway components (142). In both myocytes and murine models, the cytokine-induced activation of NF–ϰβ has been shown to inhibit muscle differentiation and function through the suppression of MyoD mRNA and protein (113). NF–ϰβ is required to up-regulate the expression of the key E3 ligases (MuRF-1 and

MAFbx/atrogin-1) which mediate sarcomeric breakdown and inhibition of protein synthesis (79). MuRF1 is up-regulated in multiple settings of muscle atrophy (83). This E3 ubiquitin ligase is responsible for mediating the ubiquitination of the thick filament of the sarcomere MyHC (71), and other thick filament components (70). MuRF-1 has also been shown to target for breakdown troponin I (279) and actin (280), which are components of the thin filament. Furthermore in the latter study it was also shown that MuRF-1 protein levels are up-regulated in muscles of non-cachectic cancer patients. The cytokine TWEAK, in particular, induces MuRF1 upregulation via NF–ϰβ, resulting in MyHC loss (267). Inhibition of classical NF–ϰβ is sufficient to decrease significantly tumour induced muscle loss, at least in Yoshida AH-130 ascites hepatoma tumour bearing rats in part, by inhibiting the up-regulation of MuRF1 (143, 281).

MAFbx/Atrogin-1 is a highly specific marker of muscle atrophy as demonstrated in other conditions causing cachexia such as immobilisation, denervation, and glucocorticoid excess (83, 84). MAFbx up regulation occurs mainly via p38 activation (116). MAFbx causes the ubiquitination of eIF3f which is directly involved in skeletal muscle protein synthesis (282). Thus MAFbx may provide a key link between the simultaneous regulation of both protein degradation and synthesis in certain models of cachexia (277). Another E3 ligase, Fbxo40 has been implicated in skeletal muscle atrophy (283). It has been suggested Fbox40 induces the ubiquitination of IRS1 therefore decreasing protein synthesis. Up regulation of Fbox40 has been seen in denervation but, at present little evidence suggests this may occur in other models of cachexia (283).

Administration of anti-murine IL-6 receptor antibody to C26 bearing mice reduced weight loss of the gastrocnemius muscle to 84% of that of control mice. The enzymatic activity of cathepsin B+L and mRNA levels of cathepsin L and poly-Ub were significantly depressed in control mice compared with C26 bearing mice, indicating that both the lysosomal cathepsin pathway and the ATP-dependant proteolytic pathway might be involved (284). The direct regulation of E3 ligase expression during IL-6-induced muscle atrophy is controversial (285). Although less evidence exists to demonstrate that IL-6 can lead directly to lipid mobilization or altered skeletal muscle protein turnover, there is general acceptance from mouse models (286) that IL-6 is produced from activated macrophages and may contribute to net muscle loss in cancer cachexia by stimulating the liver (287) to induce an acute phase protein response and subsequent reprioritisation of peripheral protein metabolism. The IL-6

receptor signals via the Janus associated kinase / signal transducer and activator of transcription (JAK/STAT) signal transduction pathway (Figure 2.2). Downstream activation results in STAT phosphorylation and translocation to the nucleus to regulate genetic transcription (102).

2.6.1.1.2 TGF-β family activating the ubiquitin proteasome pathway

A more recent factor, which has captured the attention of many investigators, is the TGF-β family member, myostatin. Genetic null animals for myostatin, demonstrate dramatic muscle hypertrophy (288). Myostatin is synthesised and secreted mainly from skeletal muscle cells. Myostatin acts firstly by signalling through the activin type II receptor, which then recruits an Alk family kinase, resulting in the activation of a SMAD2 and SMAD3 transcription factor complex (289, 290) (Figure 2.2). Secondly, Akt and downstream TORC1 pathways that promote synthesis are down regulated (289, 291). Myostatin excess causes an increase in skeletal muscle atrophy (292), whilst inhibition causes a marked increase in muscle mass and myofibre size (293-296). Myostatin antagonism has been shown to prevent cancer cachexia in the LLC mouse model (297). Activin A and B are other members of the TGF-β family that have been found to be up regulated in skeletal muscle after activation of the TNFα /TAK-1 signalling pathway (298). Both Activin A and Activin B are potent growth and differentiation factors with a broad spectrum of biological effects, including modulation of embryogenesis, neuroprotection, apoptosis, and fibrosis. These TGF-β molecules commonly bind to the ACVR2B receptor to initiate a signalling cascade leading to increased expression of MAFbx/Atrogin-1 and MuRF1 and subsequent degradation of myofibrillar proteins (79). Direct evidence linking muscle wasting to survival of the host with cancer cachexia has been lacking until recently (145, 299-301). As mentioned previously, Zhou and co-workers found C26 bearing mice that preserving muscle mass is vitally important in the organism’s survival: ACVR2B antagonism prolonged survival and prevented muscle wasting and reversed muscle loss (145). Further work demonstrated that ACVR2B pathway activation induced the ubiquitin ligases critical in muscle wasting and enhanced ubiquitination of muscle proteins, and these effects were completely abolished by ACVR2B antagonism. It was also shown that ACVR2B-SMAD signalling stimulated FOXO3 activity in muscle, which induced transcription of MAFbx/Atrogin-1 and MuRF1. Finally ACVR2B antagonism was

shown to dramatically stimulate satellite cell proliferation, which presumably also contributed to the observed rapid reversal of muscle loss in treated animals.

2.6.1.1.3 Deubiquitinating enzymes

Protein ubiquitination is a reversible process. Much attention has been focused on identification of the enzymes responsible for conjugation of ubiquitin to proteins. However, deconjugation of ubiquitin plays an important role in regulating ubiquitin-dependent pathways (302, 303). Previous studies have shown increased levels of ubiquitination of muscle proteins in several catabolic conditions including cancer (304-307). In Wistar rats bearing the Yoshida sarcoma, administration of a xanthine derivative close to pentoxifylline that inhibits TNF production, torbafylline (also known as HWA 448) prevented muscle wasting by suppressing elevated proteasome - dependent proteolysis and accumulation of Ub-protein conjugates (304). However, the deubiquinating enzyme USP 19 was shown to be overexpressed in two models of rats bearing the Yoshida sarcoma or hepatoma (308). Whether suppression of deubiquitination contributes to cancer cachexia remains unresolved.

2.6.1.2 Autophagy / lysosomal pathway

Intracellular lysosomes contain a number of enzymes required for the digestion of macromolecules, including proteases, carbohydrases, lipases and nucleases. Cathepsins L, B, D and H are the major lysosomal proteases and determine primarily the proteolytic capacity of lysosomes (100). Cathepsin-L, in particular, is induced early in catabolic states, and levels correlate with increased protein breakdown. Increased cathepsin activity has been demonstrated in various models of muscle wasting, including cancer cachexia, diabetes mellitus, dexamethasone-induced atrophy, disuse, fasting and muscular dystrophy (309, 310). The autophagy pathway is necessary to drive substrates to lysosomes, and lysosomal proteolysis is dependent on the activity of cathepsins (311). In a recent study, autophagy was shown to be increased in the muscle of mice implanted with the C26 tumour (312). In most mechanistic studies, induction of autophagy has been associated with increased activation of FOXO3A-dependent transcription of autophagy-related genes such as *LC3B* and *BNIP3* (94) (Figure 2.2). An elevation of total lysosomal protease activities

has been observed in muscles and liver of DBA/2 mice inoculated with L1210 tumour cells (313, 314) along with increased muscle levels of cathepsin L mRNA in septic rats (315). Autophagy has been demonstrated to play a role in cardiac atrophy associated with cancer cachexia (316). In the C26 tumour mouse model, autophagy (without UPP activation) in cardiac muscle was demonstrated to be responsible for loss of both myofibrillar and sarcomeric proteins. The findings in skeletal muscle in cancer cachexia models are less clear, some have shown a selective targeting of MyHC (253), whereas other studies do not support a selective sparing of MyHC (317). Furthermore, studies into variety of other catabolic conditions appear not to show a selective sparing of MyHC (318, 319).

2.6.1.3 Calpains

Several different calcium-dependent enzyme systems exist, including the calpains. The calpains are a family of intracellular, non-lysosomal, calcium-regulated cysteine proteases that mediate cleavage of specific substrates in a large number of regulatory cell processes (320). In excessive amounts, calpains are capable of degrading cytoskeletal elements, ion channels, other enzymes, cellular adhesion molecules and cell surface receptors. The ubiquitous calpains 1 and 2 (also called μ and m) have been implicated in both the initial degradation of myofibrillar proteins during muscle wasting (321) and the necrosis associated with muscular dystrophy (322). Calpains have also been implicated in the degradation of sarcomeric and cytoskeletal proteins in cultured myotubes (323) (Figure 2.2). 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 mouse hindlimb suspension model where inhibition of calpain activity preserved sarcomere structure and furthermore prevented a fall in the isometric force generating capability (324). Ubiquitous calpain activity is under the control of the endogenous inhibitor calpastatin, and downregulation of calpastatin expression has been suggested as one potential mechanism of muscle atrophy. In contrast, overexpression of calpastatin may have potential therapeutic benefits against muscle atrophy. In animal models, transgenic overexpression of calpastatin can reduce both the atrophy caused by unloading and the necrosis associated with muscular dystrophy (325). There is little evidence in the literature to allow assessment of the role of calpains in models of cancer cachexia.

2.6.2 Protein synthesis pathways

The primary role of suppressed protein synthesis in cancer cachexia has been suggested by studies with mice bearing the MAC-16 adenocarcinoma. Using L-[4-3H]phenylalanine to label proteins in the gastrocnemius muscle to determine rates of protein synthesis, a significant depression (60%) occurred in animals with a weight loss between 15 and 30% (277). In muscle 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 (326). Two major targets for reduced protein synthesis in cachexia include either inhibition of amino acid uptake or the suppression of RNA expression or translation (253). Indeed, some tumour models show as much as a 40% decrease in total muscle RNA compared with control animals (256, 327). The concept that skeletal muscle protein synthesis in cachexia is reduced simply due to anorexia alone (100) 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 (277). In the MAC-16 cancer cachexia model, catabolism and anabolism appear to occur in tandem with up to a 60% fall in protein synthesis (328). In another animal model, the Apc(Min/+) mouse, a reduction in protein synthesis seemed to precede a rise in proteolysis, suggesting differential time-dependant activation of pathways (276).

The predominant regulator of skeletal muscle hypertrophy is through stimulation of the PI3K/Akt pathway by insulin or IGF-1 (329-331) (Figure 2.2). Mice in which Akt is transgenically expressed and inducibly activated in skeletal muscle demonstrate dramatic hypertrophy upon the activation signal (332-334), helping to prove that Akt is the pathway that is sufficient to mediate hypertrophy downstream of IGF1 upregulation. Activation of Akt leads to an increase in the mTOR/p70S6K pathways and a rise in protein synthesis. As well as inducing protein synthesis, IGF1 can inhibit skeletal muscle atrophy. In the presence of up regulated IGF1 signalling, the atrophy genes MuRF-1 and MAFbx/atrogin-1 are actively inhibited (83).

2.6.3 Fat-muscle cross talk

Recent work suggests molecular cross-talk between adipose tissue and muscle that occurs through adipokines and myokines. Integrative physiology in obesity and diabetes has long emphasised the importance of chronic inflammation, increased

adipocyte lipolysis, and increased levels of circulating free fatty acids in the adipose–muscle cross-talk that contributes to lipotoxicity and insulin resistance in muscle. Intracellular accumulation of diacylglycerol triggers activation of novel protein kinases C with subsequent impairments in insulin signalling (335). A recent study in mice bearing the IL-6 producing, pro-cachectic C26 tumor showed that genetic ablation of adipose triglyceride lipase prevented the increase in lipolysis and the net mobilisation of adipose tissue associated with tumour growth (103). Unexpectedly, skeletal muscle mass was preserved and activation of proteasomal degradation and apoptotic pathways in muscle was averted. Ablation of hormone-sensitive lipase had similar, but weaker, effects. Physiologically important and previously unrecognised crosstalk between adipose tissue and skeletal muscle may therefore exist in the context of cancer cachexia (336-338).

2.7 Skeletal muscle morphology and function in cancer associated myopenia: clinical data

Results from morphological studies in humans to date are shown in Table 2.2. The limited data available suggest loss of MyHC and reduction in fibre CSA. The rectus muscle of patients with oesophago-gastric cancer cachexia (236) has been shown to lose MyHC content as well as undergo a reduction in fibre size. Similarly, the rectus muscle of patients with colorectal cancer has also been shown to lose MyHC content (339). In pancreatic cancer patients with cachexia MyHC protein levels were decreased by 45% when compared with controls (238). It has been suggested, based on MRI CSA muscle measurements and ex vivo muscle biopsies, that cancer-related cachexia is associated with a loss of muscle volume, but not of muscle function (239). After normalizing measured muscle strength for reduced CSA in cachectic patients, although muscle volume was diminished, the principal ability to generate force was maintained. The morphological basis for this finding seemed to be smaller mean fibre diameter associated with an increased total fibre number per area (239). In contrast, in a larger series of upper GI cancer patients, muscle mechanical quality measured by knee extensor strength per unit quadriceps CSA was shown to be reduced. Furthermore the degree of impairment of lower limb muscle mass, quality and function and the impact on quality of life varied with weight-loss and sex (213). Few other

studies have been conducted to resolve these findings and human data into fibre size, type, myonuclear, and protein content remain limited.

Authors	Model	Definition of cachexia	Muscle group	Myosin Fibre Type	Fibre Size (FCSA) (μm^2)	Fibre Percentage (%)
Eley (236)	Cancer (n = 15)	> 5% in 6 months	Rectus	All		↓
Weber (237)	Cancer (n = 17)	> 20% in 6 months	Quadriceps	1 2a 2x	↓ ↓ ↓	↑ ↓ ↓
Schmitt (238)	Cancer (n = 8)	> 10% in 6 months	Rectus	All		↓
Weber (239)	Cancer UGI (n = 19)	> 10% in 6 months	Quadriceps	All	↓	
Zampieri (339)	Cancer CRC (n = 10)	N/A	Rectus	2		↓

Table 2.2 - Clinical data on myosin composition of skeletal muscle in cancer cachexia

2.8 Mediators of cancer associated myopenia: clinical data

Unlike murine models where various highly specific treatments have been tested (e.g. ACVR2B receptor), there are few licensed treatments for cancer cachexia in humans and therefore the data on mediators and mechanisms are mostly of an observational nature. This naturally restricts the conclusions that can be reached. There has,

however, been a recent increase in phase II/III clinical trial activity and results from these trials promise to provide much needed insight.

2.8.1 Systemic mediators of cachexia

As in animal models, cancer cachexia in humans is considered, at least in part, to result from interactions between the host and the tumour.

2.8.1.1 The Acute Phase Response

An organism responds to the presence of acute infection, tissue injury, trauma or surgery by mounting an acute phase response (APR), this is designed to help limit tissue injury by the increased synthesis of key defence / repair proteins by the liver. However, in certain circumstances when dietary protein intake is limited and the APR is prolonged or severe, an APR can exacerbate muscle wasting by increasing the demands for certain amino acids to support increased hepatic export protein synthesis.

2.8.2 Pro-inflammatory cytokines

Pro-inflammatory cytokines, in particular IL-6, IL-1, TNF and IFN-γ, are central to systemic inflammation and the induction of an APR. Cytokine interaction between host and tumour cells within the tumour are thought to activate peripheral blood mononuclear cells (PBMC) passing through the tumour vasculature. Increased pro-inflammatory cytokine release by PBMCs has been demonstrated in cancer patients with evidence of an APR (340) (341). Moreover, patients with pancreatic cancer and cachexia appear to have higher intra-tumoural IL-6 expression and this is associated with increased PBMC IL-6 production (342). Tumour associated macrophages (TAM) may be one important cellular sub-type involved in the intra-tumoural interaction between host and tumour cells (343). In cancer, host mononuclear cells are recruited to tumours by various signals including hypoxia. 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 (343).

Despite the pivotal role of pro-inflammatory cytokines in the aetiology of cancer cachexia in animal models, human trials to date using cytokine antagonists have not generally been successful at ameliorating muscle loss. The use of infliximab, an anti-TNF-α monoclonal anitibody (mAb), in conjunction with docetaxel was shown to be associated with increased fatigue and worsened QoL scores in NSCLC patients compared with docetaxel and placebo, and therefore the trial was stopped (344). Neither arm of the study demonstrated attenuation of weight loss. A further trial of infliximab with gemcitabine in pancreatic cancer patients was unable to demonstrate a significant improvement in LBM or survival (345), whereas a trial of etanercept, a TNF-α inhibitor, in cachectic cancer patients was not associated with weight gain or improved survival (346). Trials of pentoxyfilline, a cytokine inhibitor, have also shown little clinical benefit in the treatment of cachexia and anorexia in cancer patients (347). In human trials of an anti-IL6 mAb early clinical studies in patients with non-small-cell lung cancer has shown it to be safe and well tolerated. Treatment with an anti-IL6 mAb (BMS-945429) improved lung symptoms, reversed fatigue, and a trend towards a decrease in the loss of lean body mass was noted (348). In another study of patients with cholangiocarcinoma, selumetinib (MAPK1 and IL-6 secretion inhibitor) was shown to promote a gain in muscle mass (349).

2.8.3 Other circulating mediators

2.8.3.1 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 (350). In sarcopenia, women with low vitamin D have been shown to have approximately twice the risk of reduced muscle mass and strength and a similar relationship was observed with raised parathyroid hormone levels (351). Vitamin D deficiency has been reported in over 80% of cancer patients (352) and may be particularly prominent in patients receiving chemotherapy (353). A retrospective study of advanced cancer patients reported 70% vitamin D insufficiency and an association with anorexia and fatigue (354). In cachectic cancer patients, vitamin D gene receptor polymorphisms have been linked to a more aggressive form of

cachexia (205). Although these findings suggest a role for vitamin D in cachexia, studies directly linking it with muscle mass in cancer cachexia patients are lacking.

2.8.3.2 Glucocorticoids

In cachectic pancreatic cancer patients, elevated serum levels of cortisol have been shown, along with elevated cortisol:insulin ratios (37). Glucocorticoids are administered frequently to cancer patients undergoing chemotherapy or radiotherapy. This cancer therapy is often associated with exacerbation of muscle wasting. Glucocorticoid atrophy seems specific to type II fibres. Mechanism may involve upregulation of protein degradation pathways, upregulation of myostatin and enhanced glutamine synthetase activity (355). Glucocorticoids inhibit physiological secretion of growth hormone and appear to reduce IGF-1 activity in target organs.

2.8.3.3 Insulin

Insulin can stimulate protein synthesis via the PI3K/Akt pathway. Both low insulin production and peripheral insulin resistance has been demonstrated in cancer cachexia (100). In cancer patients, treatment with low dose insulin therapy as part of a combination therapy regimen resulted in a rise in carbohydrate intake, and increase in body fat, and a fall in serum-free fatty acids. Despite these findings however, there was no change in lean body mass, maximum exercise capacity or spontaneous physical activity (356). Higher serum insulin did not change IGF-I levels, which may explain the dissociated effects by insulin on whole body fat and lean tissue.

2.8.3.4 Ghrelin

A randomised controlled trial has investigated the effects of intravenous ghrelin taken at both low dose and high dose in patients with a variety of advanced metastatic cancer. No difference between treatments was seen for nutritional intake, symptoms, adverse effects or tolerability (357). In another study, 31 patients with metastatic gastrointestinal cancer were randomised in a double-blind manner to ghrelin at 2 different doses subcutaneously daily for 8 weeks. In the high-dose group (versus the low-dose group) appetite scores were significantly better, and there was a trend to

less fat-free mass loss and improved energy balance. There were no differences in food intake, quality of life or physical activity (358). The studies to date are small phase I and phase II trials, and therefore, results should be treated with caution. A phase III, randomised, placebo-controlled clinical trial assessing anamorelin hydrochloride in patients with non-small-cell lung cancer-associated cachexia is currently recruiting patients (clinicaltrails.gov/NCT01505764).

2.8.3.5 Androgens

Testosterone inhibits release of TNF, IL-1, IL-6 from macrophages and stimulates production of IL-10 (359-361). 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 (362). In males with pancreatic cancer, systemic inflammation was associated with hypogonadism which in turn was associated with shortened survival (214). Despite these findings, trials have not been able to show an improvement with exogenous androgens. A trial of nandrolone decanoate in patients with advanced NSCLC demonstrated a trend for decreased weight loss (363). In another study comparing fluoxymesterone with megestrol acetate or dexamethasone, fluoxymesterone was inferior in terms of appetite and weight gain (364). Recently, SARMs have received much attention as potential muscle-targeted treatments for cancer cachexia. In humans, Phase I and II clinical trials have shown that SARMs increased LBM and enhanced functional status (365). In a recent phase IIb randomised, double blind, placebo controlled study, treatment with enobosarm (GTx-024) was shown to significantly increase lean body mass and muscle function in 120 healthy elderly men and women (366) At present, enobosarm is being assessed in a randomised, placebo-controlled, phase III clinical trial in patients with non-small-cell lung cancer receiving first-line chemotherapy treatment (clinicaltrails.gov/NCT01355497).

2.8.3.6 Myostatin

In a recent study of myostatin expression in muscle biopsies from non weight losing lung and gastric cancer patients, there was evidence of increased expression in the gastric cancer patients (301). This suggests that in human cancer cachexia,

myostatin alterations may vary by primary tumour site. There are at least two ongoing phase II trials of anti myostatin strategies (clinicaltrials.gov/NCT01505530), (clinicaltrails.gov/NCT01433263).

2.8.3.7 Dietary factors

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid found in fish oil. Polyunsaturated fatty acids have been shown to reduce cancer cachexia (367). EPA causes a reduction in the production of pro-inflammatory cytokines in patients with cancer as well as PIF. A number of trials demonstrated preservation of LBM, increased physical activity, improved appetite and weight gain with taking either fish oil or EPA alone in weight-losing cancer patients (368-371). However phase III, large, randomised clinical trials failed to show the benefit of EPA over placebo on LBM (372-374). However, in the last 2 years, several studies have again pointed towards potential benefits of EPA for attenuating LBM loss, as well as maintenance or gain of LBM. Megastrol acetate, a steroidal progestin and progesterone derivative with appetite stimulating properties and the anti-inflammatory, ibuprofen have been given together and observed to increase body weight and lead to a reduction in C-reactive protein concentrations (375). Megastrol acetate, in conjunction with formoterol has also been shown to improve muscle mass and function (376). More research is needed on drugs such as eicosapentaenoic acid as well as focusing on multimodal approaches combining drugs and non-drug interventions.

2.9 Mechanisms of cancer associated myopenia: clinical data

2.9.1 UPP / E3 ubiquitin ligases

In many acute models of cachexia, including cancer, the UPP is thought to be fundamental in the process of muscle atrophy (83, 84). However, human investigations have failed to be conclusive. Studies including patients in ICU 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 (85-88, 377-379). Likewise, investigations of UPP activity in quadriceps muscle biopsies have shown similar levels to healthy controls in patients with lung cancer and weight loss <10% (termed pre-cachexia by the authors) (380). In a transcriptomic study of UGI cancer patients, candidate genes including FOXO and ubiquitin E3 ligases, were not related to weight loss. Furthermore, promoter analysis identified that weight loss-associated genes had fewer FOXO binding sites than expected by chance (89) (Table 2.3). 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 a suggestion that the lysosomal pathway was increased (97) (Table 2.3). In contrast, in gastric cancer patients with average weight loss 5.2%, increased UPP activity (determined by measurement of RNA and cleavage of specific fluorogenic substrates) was seen compared with controls, with a further effect with increasing tumour stage, weight loss and lower albumin (381) (Table 2.3). The same group also demonstrated higher levels of ubiquitin mRNA in gastric cancer patients compared with controls, but there was no relationship with weight loss. This led the authors to conclude that the UPP was activated early in disease before there was overt clinical evidence of cachexia (382). 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 (383) (Table 2.3). The authors 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 (384) (Table 2.3) and the MAC-16 murine cachexia model (227). However, in these studies, UPP activity seemed to increase after a threshold of ~10% weight loss with a peak between 12-19% weight loss.

	Tumour type	% Weight loss	UPP mRNA	Proteasome activity	Calpain activity	Cathepsin B mRNA
Williams (383)	GI	0 – 10%	↑			
Bossola (381)	GI	>5%		↑		
Khal (227)	GI	>10%	↑			
Stephens (89)	GI	>5%	=			
Smith (385)	GI	0 – 5%	=		↑	
Jagoe (97)	Lung	<5%	=			↑

Table 2.3 – Findings of different studies in humans on potential mechanisms of skeletal muscle protein degradation

2.9.2 Autophagy / Lysosomal pathway

Human studies have reported increased cathepsin D enzyme activity in cancer patients (96) and cathepsin B mRNA in lung cancer patients (97) Table 2.3. 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 (97) Table 2.3. This perhaps has some appeal because, although one study detected radio-labelled myofibrillar proteins in lysosomes (386), there is other evidence to suggest that lysosomal proteolysis in isolation is not sufficient to degrade myofibrillar proteins (70, 387, 388).

2.9.3 Calpains

Calpains are capable of degrading cytoskeletal elements, ion channels, other enzymes, cellular adhesion molecules and cell surface receptors. Furthermore, calpains cleave the enzyme xanthine dehydrogenase to xanthine oxidase, thus indirectly creating the formation of superoxide radicals. Calpains have been implicated in the degradation of sarcomeric and cytoskeletal proteins in cultured myotubes (323). Gastric cancer patients with minimal weight loss (average 1%), had an increase in rectus 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 (385) Table 2.3.

2.9.4 Caspases

Caspases are a group of enzymes usually associated with the induction of cellular apoptosis in response to death signals. There is some evidence for a role for apoptosis in muscle wasting experienced by cachectic patients. Muscle biopsies from weight-losing patients with upper GI cancer showed a three-fold increase in muscle DNA fragmentation compared with control subjects (389). Furthermore, the increase in DNA fragmentation was associated with a four-fold increase in poly (adenosine diphosphate-ribose) polymerase (PARP) cleavage, indicating the presence of

apoptosis (389). No human studies to date have focused on targeting caspases or apoptosis in the prevention of cancer cachexia.

2.9.5 Myogenesis

Decrease in skeletal muscle regeneration may also contribute to the loss of skeletal muscle mass in cancer cachexia. In both health and disease, skeletal muscle is able to undergo an extensive repair process aimed at preventing the loss of muscle mass via activation of satellite cells (390) (391). The satellite cell starts off as a myogenic precursor during embryonic development, and upon muscle injury starts proliferating with a variety of genes being regulated. Myogenin is subsequently up-regulated leading to terminal differentiation, followed by a permanent exit from the cell cycle. In a recent study looking at patients with gastric cancer these genes were found to be up regulated compared with a control group (392), whether this suggests potential for muscle regeneration during cancer cachexia is unknown.

2.10 Future prospects

The heterogeneity between morphology, mediators, and mechanisms of animal models and human cancer cachexia is vast. There are some comparisons that can be drawn from the two but it must be realised that these may represent distinct disease processes. The relatively acute wasting seen in young, metabolically active animal models may have little bearing on the chronic disease patho - physiology seen in cancer patients with chronic wasting. Progress in treating the human form of cancer cachexia can only move forwards through carefully designed large randomised controlled clinical trials of specific therapies with validated biomarkers of relevance to underlying mechanisms. The following Chapters will try to elucidate some of the morphology, mediators, and mechanisms underlying the human form of cancer cachexia to provide a robust phenotype to consider in a genetic analysis of patients with cancer cachexia. Chapter 4 looks at the clinical classification of cancer cachexia and demonstrates phenotypic correlates in human skeletal muscle.

CHAPTER 3

Materials and Methods

3.1 Ethical approval

All studies contained in this thesis received full ethical permission from their respective host institutions. Ethical approval for research carried out in Edinburgh was granted by the Lothian Research Ethics Committee (reference 06/S1103/75 for the phenotyping study and 08/S1103/19 for the genotyping study, see appendix)

3.2 Study participants

3.2.1 Clinical Classification of Cancer Cachexia: Phenotypic Correlates in Human Skeletal Muscle study (Chapter 4)

Patients were recruited to this study between August 2007 and October 2010. All patients were above 18 years of age had a diagnosis of upper gastro-intestinal cancer (oesophageal, gastric, pancreatic) and were undergoing surgery with the intent of resection of the primary tumour. All patients were enrolled before any treatment was initiated and within 1-2 weeks of diagnosis. Newly diagnosed patients were identified principally from the regional oesophago-gastric and hepatobiliary cancer multidisciplinary team (MDT) meetings at the Royal Infirmary of Edinburgh, UK. The oesophago-gastric and hepatobiliary MDTs provides regional referral services for patients with oesophageal, gastric and pancreatic cancer from the Lothian and Borders regions. Patients with newly diagnosed upper gastro-intestinal cancer within these regions are referred to the MDTs which meet on a weekly basis to discuss all new referrals and to decide on individual patient management strategies. Participants were recruited at their surgical clinic appointments at the Royal Infirmary of Edinburgh. All procedures were approved by the NHS Lothian local research ethics committee. The study conformed to the standards set by the Declaration of Helsinki.

3.2.2 Genetic signatures associated with susceptibility to cancer cachexia vary according to weight-loss or low muscle mass phenotype (Chapter 6)

The main study cohort was recruited for this study between January 2004 and November 2012 from the following six institutions:

1) NHS Lothian, Edinburgh, UK

2) Cross Cancer Institute, Edmonton, Canada

3) McGill Cancer Center, Montreal, Canada

4) Palliative Research Centre, Norwegian University of Science and Technology (NTNU), Norway

5) Cantonal Hospital, St Gallen, Switzerland

6) Department of Medical Oncology, University Hospital of Larissa

All subjects participated in clinical or research studies on cancer cachexia at the host institutions under ethically approved protocols allowing for analysis of patients' DNA. Recruitment was conducted at first presentation (treatment naive) to surgical, oncology or palliative care clinics at each institution. Recruitment was performed sequentially with the following exclusion criteria: (i) under 18 years of age; (ii) cognitive impairment; (iii) inability to give written, informed consent; (iv) presence of underlying infection; (v) on corticosteroids. Patients recruited generally had cancer types with propensity to develop cachexia (e.g. gastric/oesophageal, pancreatic, lung).

NHS Lothian

All patients recruited had either a confirmed diagnosis of gastro-oesophageal or pancreatic malignancy. Approximately 430 retrospective patients recruited for previous studies with blood stored in a tissue bank with relevant phenotypic data were entered into the study. These patients were identified principally from the regional oesophago-gastric and hepatobiliary cancer multidisciplinary team (MDT) meetings at the Royal Infirmary of Edinburgh, UK. Patients were recruited at first presentation to surgical or oncological clinic at Royal Infirmary of Edinburgh or Western General Hospital, Edinburgh, UK.

Cross Cancer Institute

Approximately 190 patients were entered into the study. All patients had been previously recruited for clinical research studies with relevant phenotypic data and had blood stored in a tissue bank. All patients had a confirmed diagnosis of non-small cell lung cancer (NSCLC).

McGill Cancer Center

All patients recruited had confirmed diagnosis of colorectal, pancreatic or non-small cell lung cancers. Patients had previously participated in clinical research studies with relevant phenotypic data and had blood stored in a tissue back. Approximately 260 patients were entered into the study.

Norway Palliative Research Centre

Approximately 245 patients were entered into the study. All patients had been previously recruited for clinical research studies with relevant phenotypic data and had blood stored in a tissue bank. All patients had a confirmed diagnosis of lung cancer.

Cantonal Hospital

All patients were recruited from Oncology & Palliative Medicine, Cantonal Hospital, St. Gallen, Switzerland from January 2007 to December 2008. Patients with all proven malignancies were considered and 84 patients were retrospectively recruited.

Larrisa Department of Medical Oncology

All patients were recruited from Department of Medical Oncology, University Hospital of Larissa, Greece from January 2010 to December 2011. Patients with all proven malignancies were considered and 67 patients were prospectively recruited.

Newly recruited patients

Out of the patients recruited for this study (n=1276), 731 were used in a previous study identifying SNPs associated with the genetics of cancer cachexia. Further phenotyping including identification of CT scans used to compute levels of muscularity were obtained for these subjects. A further 545 patients were recruited for use in the current genotyping study with phenotyping including identification of CT scans.

3.3 Clinical and pathological data collection

Demographic data was recorded and included age and sex for each patient. The date of diagnosis was also recorded in all instances.

Histological confirmation of disease was obtained where possible and patients were staged according to the American Joint Committee on Cancer stage groupings (AJCC). Tumours of the gastro-oesophageal junction were classified according to Siewert and Stein and those classified as type I and II were staged as oesophageal tumours and type III as gastric cancers.

3.4 Nutritional assessment (Chapter 4 and 6)

All patients underwent measurements of height and weight at the time of recruitment. Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. There is evidence to support the reliability of self-reported weight and weight history (Perry et al, 1995; Stunkard & Albaum, 1981). Individual weight loss was calculated and expressed as percentage of pre-morbid body weight lost. Height and weight data were subsequently used to compute a common anthropometric descriptor, body mass index (BMI) (kg/m^2).

3.5 Collection of biological samples (Chapter 4 and 6)

3.5.1 Blood collection (Chapter 4 and 6)

Patients recruited for the phenotypic definition and candidate gene studies had up to 10ml of blood collected into EDTA tubes and tubes with gel separator and clot activator. The blood was usually collected when the patients were having blood taken as part of their on-going clinical care as requested by their supervising clinician and not merely for participation in the research study.

3.5.2 Rectus abdominis muscle biopsy and storage for biochemical analysis (Chapter 4 and 6)

For all patients recruited in the phenotypic study and the transcriptomic part of the genetics study, biopsies were taken at the start of open abdominal surgery under general anaesthesia. Patients had fasted overnight prior to surgery. The edge of the rectus abdominis was exposed and a $1cm^3$ specimen removed using sharp dissection. The biopsy was cleaned of gross blood contamination. Obvious fat/fibrous tissue was removed prior to placement in a cryotube and being snap frozen in liquid nitrogen and stored at -80°C.

3.5.3 Rectus abdominis muscle sample preparation for cryo-section (Chapter 4)

For patients recruited for the phenotypic definition study, a 0.1 – $0.5cm^3$ section of muscle was cut. Liquid nitrogen was used to cool isopentane solvent in a tube to a temperature of ~-190°C. The section of muscle was stitched onto a segment of cork. OCT solution was placed at the junction between the cork base and the muscle. This was then lowered with the cork uppermost (i.e. muscle first) into cooled solvent and held for approximately 5 minutes (until the muscle was frozen). Samples were then stored at -80°C until use.

3.6 Blood assays (Chapter 4 and 6)

3.6.1 Measurement of C-reactive protein (CRP)

Serum CRP concentration was measured using an automated immuno-turbidimetric assay. Using this assay, a concentration of 10mg/l represents the upper limit of normal range with most healthy individuals having a serum concentration <2mg/l.

3.7 Body composition analysis (Chapter 4 and 6)

3.7.1 Estimation of total body skeletal and adipose tissue using computed tomography (CT) scans

Digitally stored CT images completed with a spiral CT scanner for initial staging and routine diagnostic purposes were analysed using semi-automated software which permitted specific tissue demarcation using Hounsfield unit thresholds of –29 to +150 for skeletal muscles (393), –150 to -50 for visceral adipose tissue (394), and –190 to –30 for subcutaneous adipose tissue (393). Cross-sectional areas (cm^2) are computed for each tissue by summing tissue pixels and multiplying by the pixel surface area. A transverse CT image from the third lumbar vertebrae (L3) was assessed for each scan date and tissue volumes estimated (395) (Figure 3.1). The muscles in the L3 region contain psoas, erector spinae, quadratus lumborum, transversus abdominus, external and internal obliques, and rectus abdominus. All CT images were analysed by a single trained observer, tissue boundaries were manually corrected as needed. Cross-sectional area for muscle was normalized for stature (cm^2/m^2). Cut offs for low muscularity were either based on a CT-based sarcopenic obesity study of cancer patients by Prado et al. (i.e., L3 skeletal muscle index: ≤38.5 cm^2/m^2 for women and ≤52.4 cm^2/m^2 for men) (28) (used in Chapter 4), or an updated CT-based sarcopenic obesity study of cancer patients by Martin et al. and adjusted for BMI (396) (used in Chapter 6) (Figure 3.2).

Estimates of whole body stores were generated from the raw data (cm^2) using the regression equations by Mourtzakis et al. (397), which show a close correlation between muscle and fat areas in CT images at the third lumbar vertebrae and whole body compartments of fat-free mass (FFM) and fat mass (FM) respectively.

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

The respective indexes for FFM and FM (kg/m^2) were calculated.

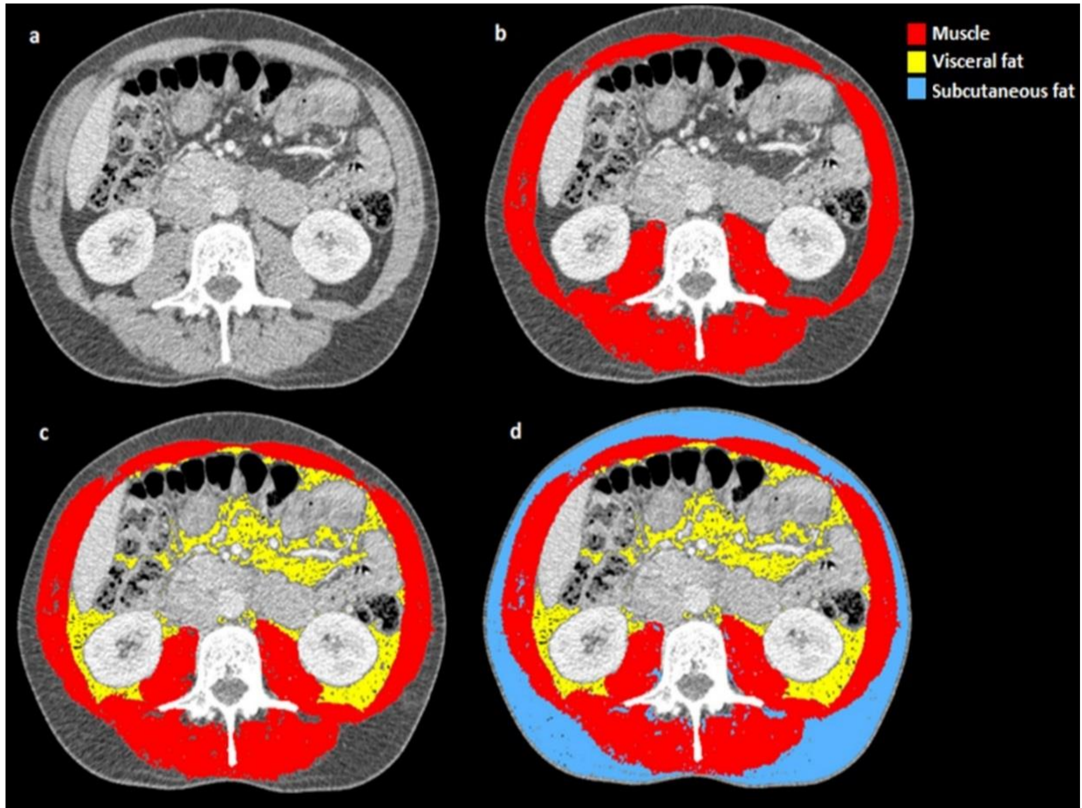


Figure 3.1 - Cross sectional CT images at L3 showing demarcation of skeletal muscle and adipose tissue using Slice-o-matic software

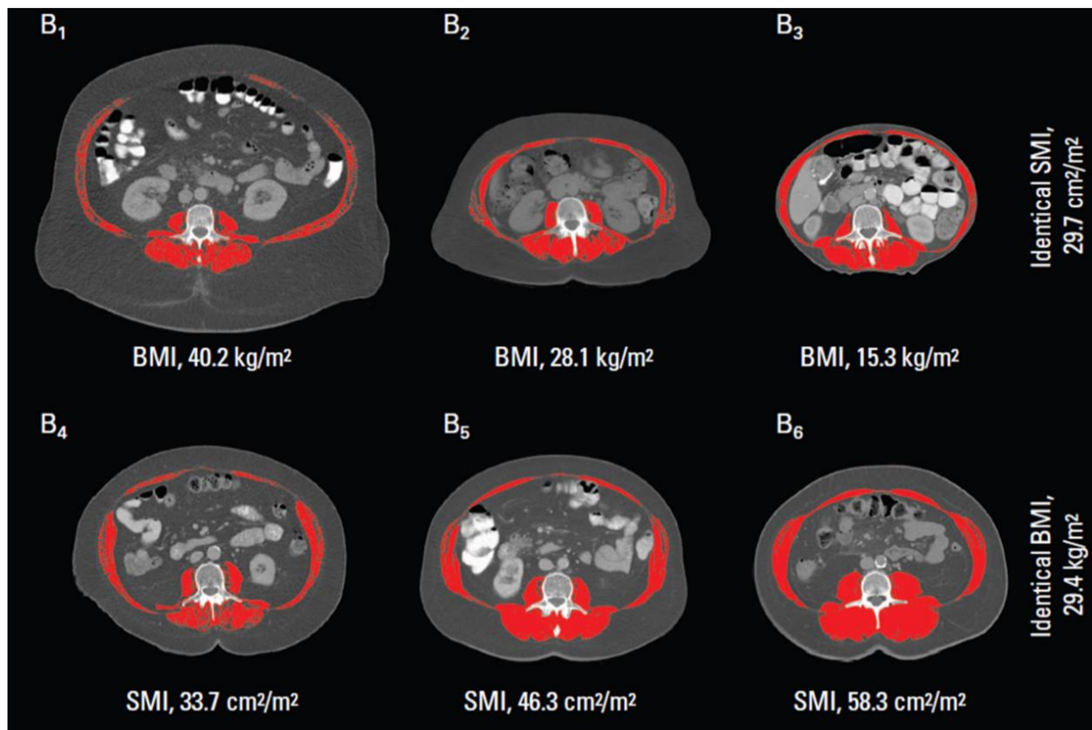


Figure 3.2 - Skeletal muscle depletion is a powerful prognostic factor, independent of BMI. Panels B_{1-3} highlight variation in BMI for female patients with identical SMIs (29.7 cm^2/m^2) and different BMIs. Panels B_{4-6} highlight variation in SMI for overweight female patients with identical BMIs (29.4 kg/m^2) and different SMIs (adapted from (396).

3.8 Cellular methods (Chapter 4 and 6)

3.8.1 Immunohistochemistry (Chapter 4)

The frozen muscle sections were co-stained for laminin (L9393, Sigma-Aldrich, Buchs, Switzerland) and myosin heavy chain type I or IIa to distinguish each fibre type (BA-D5 for type I, SC-71 for type IIa). The paraffin sections were stained for phospho-STAT3 (D3A7, Cell Signaling Technologies, Danvers, MA, USA) with a Ventana discovery XT (Roche group, Tucson, USA). Images of the entire tissue section were acquired using a VS120 slide scanner (Olympus Corporation, Tokyo, Japan). The distribution of myosin heavy chain fibre types, the cross section area of the individual fibres in the section, and the phospho-STAT3 positive nuclei and staining density were analysed using the proprietary image analysis platform ASTORIA (Automated Stored Image Analysis) developed by Novartis/Preclinical Safety.

3.8.2 Tissue Preparation for DNA, RNA and Protein extractions (Chapter 4)

Skeletal muscle tissue was minced and ground on dry ice. Aliquots were weighed using an analytical balance (Mettler Toledo) and stored at -80°C until use.

3.8.3 DNA and RNA extraction and linearity of the extraction method (Chapter 4)

DNA and RNA from human skeletal muscle tissue was extracted and purified with the automated Maxwell 16 system (Promega, Duebendorf, Switzerland). To determine the linearity of the extraction methods using the Maxwell 16 system, DNA and RNA was extracted from 4mg, 6mg, 8mg, and 10mg of muscle, respectively. Calculating the total DNA and RNA content per wet weight (which in a linear extraction system should be equal for all aliquots), allowed us to define the linear range of the Maxwell 16 extraction system. Based on these preliminary studies, aliquots of 4-8mg human skeletal muscle tissue were used for all subsequent DNA and RNA extractions. Using more starting material drastically reduced the total DNA and RNA content per wet weight (data not shown).

For DNA extraction, the Maxwell 16 LEV Blood DNA Kit (Promega) was used with a slightly adapted protocol compared with the manual's instructions. Briefly, 300µl of Tail Lysis Buffer from the kit ReliaPrep gDNA Tissue Miniprep System (Promega) was added to minced and ground human skeletal muscle tissue in Precellys 24 lysing kit tubes. Tissue was further homogenized using the high-throughput homogenizer Precellys 24, for 10s. After cooling on ice for 5 minutes, 30µl of the protein K and 5µl of the 1-Thiolglycerol solution were added. This mixture was incubated at 56°C for 2 hrs. Afterwards, the lysate was transferred into well 1 of the LEV Blood DNA cartridge, and diluted with 300µl nuclease-free water. For the elution, 50µl of elution buffer was added into elution tubes. The Maxwell 16 instrument was started using the DNA Blood program.

For RNA extraction, the Maxwell 16 LEV simplyRNA Tissue Kit was used (Promega), following the manual's instructions. Briefly, minced and ground human muscle tissue was incubated in 200µl of chilled 1-Thioglycerol/Homogenization solution and further homogenized using the Precellys 24 system (see DNA). Afterwards, the samples

were heated at 70°C for 2 min, then the lysates were allowed to cool down. 200µl of lysis buffer was added to the cooled-down homogenate, mixed vigorously, followed by transfer of the total 400µl into well 1 of the Maxwell 16 LEV cartridge. 5µl of DNAse was added to well 4 of the cartridge and, 50µl RNAse-free water was added to 0.5ml Elution Tubes and the RNA extraction program was started at the Maxwell 16 instrument.

Extracted DNA and RNA were measured spectrometrically using a Trinean DropSense Instrument (Trinean, Gentbrugge, Belgium) for quantity and quality.

3.8.4 Protein extractions (Chapter 4)

To extract proteins, 300 µl of PhosphoSafe Extraction Reagent (Millipore) was added to a specific amount (between 8 and 18mg) of homogenized human skeletal muscle tissue. To further homogenize the samples, the Precellys 24 system was used (see section above). After incubation on ice for 5 min, the lysates were spun at 800xg for 5 min at 4°C. Supernatants were transferred into new tubes and spun for another 12 min at 1600xg at 4°C. Supernatants were collected and protein concentrations measured using the BCA Protein Assay Kit (Pierce) with BSA as a standard. Afterwards, phosphatase inhibitor cocktail (Roche) was added and the samples were stored at -80°C until further use.

3.8.5 Western blots (Chapter 4)

20 µg of human skeletal muscle protein extracts (see above) in reducing Laemmli SDS sample buffer were boiled for 5 min at 95°C and then separated by SDS-PAGE on 4-20% gradient gels (Bio-Rad, Cressier, Switzerland), blotted to Nitrocellulose membranes (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad), blocked for 1h in 5% non-fat milk in Tris-buffered saline+0.05% Tween-20, incubated overnight with primary antibody, rinsed, and incubated for 1h with peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Santa Cruz, Heidelberg, Germany) at room temperature. Blots were developed using ECL (Roche, Rotkreuz, Switzerland) or SuperSignal West Femto substrate (Thermo Scientific, Wohlen, Switzerland) and exposed to Kodak film (Kodak, Rochester, NY, USA).

Rabbit monoclonal antibodies used were: Beclin-1 (clone D40C5), Atg5 (clone D1G9) , Atg7 (clone D12B11), Atg12 (clone D88H11), SMAD3 (clone C67H9), phospho-NFκB p65 (Ser536) (clone 93H1) and α-tubulin (clone 11H10) (all from Cell Signaling Technologies, Danvers, MA, USA), phospho-SMAD3 (Ser423/Ser425, clone EP823Y) (Millipore, Billerica, MA, USA). Rabbit polyclonal antibodies used were: Gelsolin (Cell Signaling Technologies).

Western blots were analyzed densitometrically using ImageJ software version 1.45 (NIH, Bethesda, MD, USA; http://rsbweb.nih.gov/ij). Band intensity of each sample was normalised to that of α-tubulin.

3.8.6 DNA extraction from blood (Chapter 6)

Genomic DNA was extracted from samples of blood taken in EDTA tubes. The Wizard Genomic DNA purification kit (Promega, Southampton, U.K) was used. Whole blood was centrifuged at 1,500g for 5 minutes at 4°C. 300 µl of the cellular layer was added to 900 µl of cell lysis solution in a 1.5 ml micro centrifuge tube and mixed thoroughly by inverting the tubes several times. The mixture was incubated for 10 minutes at room temperature followed by centrifugation at 15,000g for 20 seconds also at room temperature. The supernatant was discarded and the process of adding cell lysis solution, incubation and centrifugation was repeated once more. 300 µl of nuclei lysis solution was added to the pellet and the tube was vortexed to resuspend the white cells. The solution was pipetted gently several times to lyse the white cells. 1.5 µl of RNase solution (Promega) was added to each tube, mixed and incubated at 37°C for 15 minutes. After allowing the tubes to cool to room temperature, 100 µl of protein precipitation solution was added and mixed by vortex for 15 seconds. Samples were then centrifuged at 15,000g for 3 minutes at room temperature. The supernatants were then transferred to a clean 1.5 ml micro centrifuge tube and mixed with 300 µl of pure isopropanol. Gentle mixing reveals the appearance of the DNA as white thread-like strands. Further centrifugation at 15,000g for 1 minute at room temperature allows the DNA to be collected as a pellet at the bottom of the tube. The supernatant in then decanted and 300 µl of ethanol 70% is added to wash the pellet. The samples were then centrifuged again for a further minute at 15,000g and the ethanol carefully aspirated away. 100 µl of DNA rehydration solution (10 mM Tris-HCl

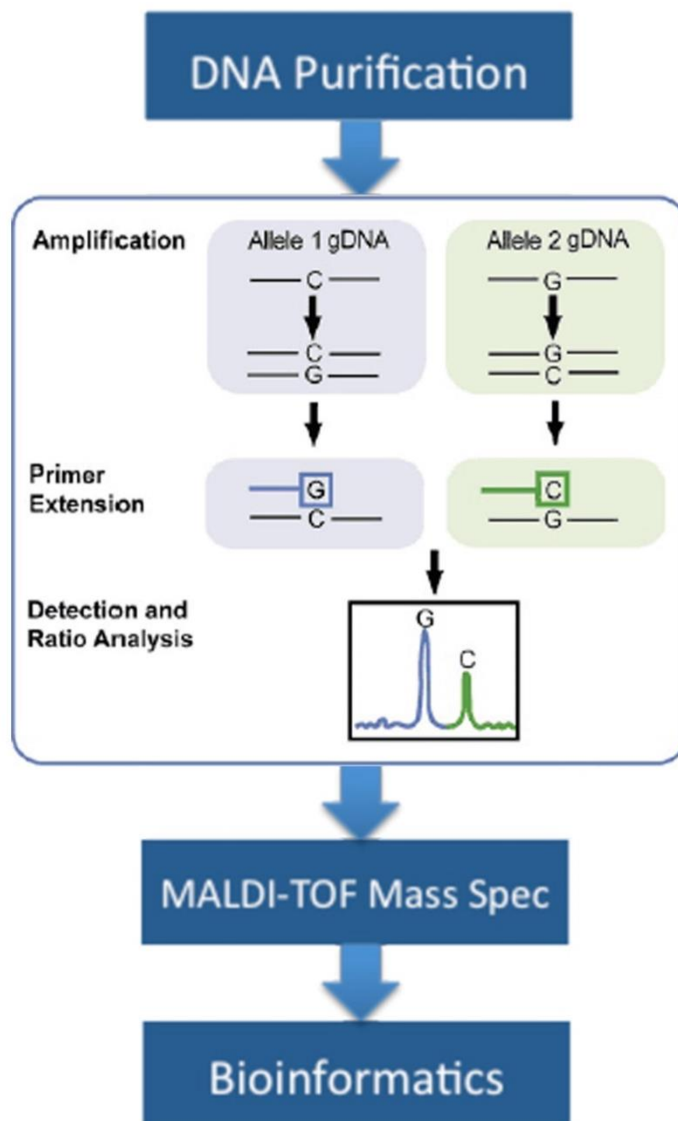
and 1 mM EDTA) was added to each tube and samples were incubated at 65°C for 1 hour. DNA samples were then stored at 4°C.

3.8.7 Quantification of DNA (Chapter 6)

DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer. Absorbance at 260nm were used for quantification of nucleic acids, optical density of 1 corresponding to 50ng/µl DNA. Absorbance ratios (260 nm/280 nm) of approximately 1.8 for DNA indicated that the nucleic acid preparations were sufficiently free from protein contamination for downstream experiments.

3.9 Single nucleotide polymorphism (SNP) genotyping (Chapter 6)

Genotyping of the samples were performed on the Sequenom iPLEX Gold platform (San Diego, CA, USA) or TaqMan assay (for rs4280262) using services from the McGill University and Genome Quebec Innovation Center, Montreal, Quebec, Canada. The iPLEX assay is based on a simple single-base primer extension assay. A protocol overview is provided in Figure 3.3. Polymorphisms selected were validated for assay feasibility using DNA from apparently healthy Caucasian subjects (n=92) from Coriell Institute (Coriell Panel, Coriell Institute of Medicine, CA, USA) (398). Deviations from Hardy-Weinberg equilibrium (HWE) were assessed in the Coriell panel of control population using the χ^2 test with 1 degree of freedom (df); a p-value of <0.001 was considered as a significant deviation from the HWE proportions.



1) The iPLEX assay is truly homogeneous, in the sense that after PCR, reagents are added to the reaction cocktail in a three step protocol. No transfer or pipeting into different reaction containers is required.
2) PCR primers are designed in a region of approximately 100 base pairs around the SNP of interest and an extension primer is designed immediately adjacent to the SNP. The assay design is performed in a highly automated fashion by the AssayDesigner software module.
3) The starting point of the iPLEX assay is PCR amplification, followed by the addition of Shrimp Alkaline Phosphatase (SAP) to inactivate remaining nucleotides in the reaction.
4) Following a brief incubation, the primer extension mixture is added and conducted using a standardized cycling program.

5) Finally, CleanResin is added to the mixture to prepare it for deposition on a 384-well SpectroChip®.
6) SpectropChips enable an automated readout and data analysis by a Compact TM MALDI-TOF mass-spectrometer. Data analysis is performed on the Typer Software Module

Figure 3.3 - Work flow for genotyping using the Sequenom iPLEX Gold platform (Adapted from iPLEX® Sequenom protocol) for processing and genotyping patient samples from Chapter 6

3.10 Microarray analysis (Chapter 6)

Total RNA was isolated using Trizol (Sigma-Aldrich, Oakville, ON, CAN), purified using Qiagen RNeasy columns (Mississauga, ON, CAN), quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its integrity evaluated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocols. All RNA samples had RNA Integrity Numbers (RIN) greater than 7.0.

RNA was subjected to linear amplification and Cy3 labeling and hybridization to Agilent Whole Human Genome Arrays using Agilent kits (One Color Low RNA Input Linear Amplification Kit Plus, One Color RNA Spike-In Kit and Gene Expression Hybridization Kit). The arrays were scanned using an Agilent Scanner, the data was extracted and quality was evaluated using Feature Extraction Software 10.5.1 (Agilent). The data was normalized using GeneSpring GX 11.5.1 (Agilent) (Figure 3.4 and 3.5). The data used in this publication have been deposited in the U.S. National Center for Biotechnology Information (NCBI) Gene Expression Omnibus25 and are accessible through GEO series accession number GSE41726.

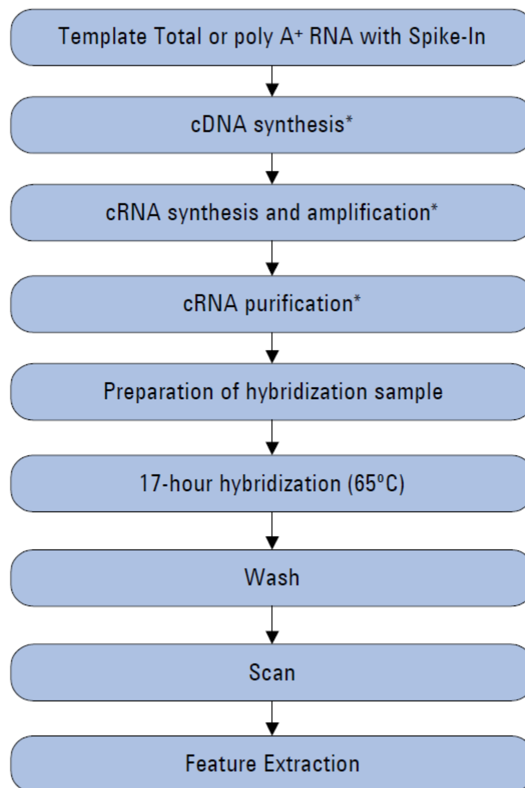


Figure 3.4 - Work flow for sample preparation and array processing to quantify gene expression in patient samples from Chapter 6.

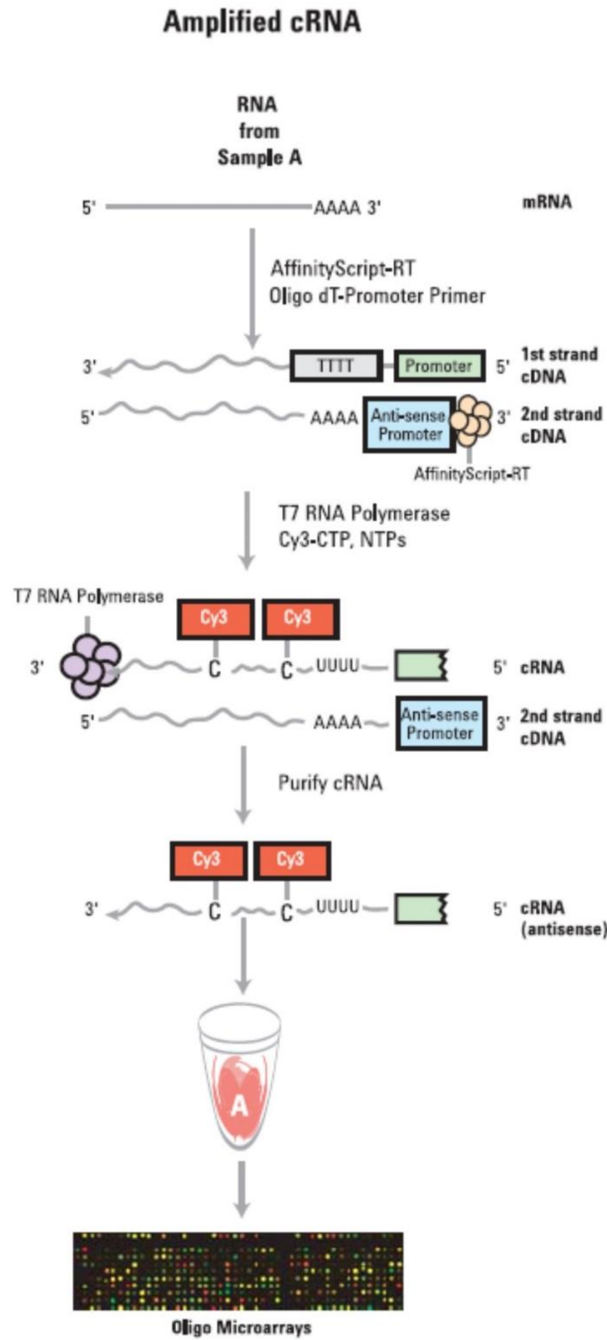


Figure 3.5 Schematic of amplified cRNA procedure. Generation of targets for a one-color microarray experiment, the Cy3-labeled “A” sample was produced and hybridized to quantify gene expression in patient samples from Chapter 6.

3.11 Statistics

3.11.1 General statistics

General statistical analyses were performed using SPSS® Version 20.0 (SPSS, Chicago, Illinois, USA). Data are presented as mean ± standard deviation unless otherwise stated. In Chapter 4 comparisons between groups were performed using unpaired Student's t tests, whereas possible relationships were evaluated using Pearson's correlations. Results were considered significant if p values were less than 0.05.

3.11.2 SNP genotyping statistical analysis (Chapter 6)

Statistical analyses were performed using PLINK (version 1.06) (399). Unconditional logistic regression was employed to calculate ORs and their 95% CI for the minor allele of individual SNPs and its association with each proposed cachexia phenotype. All analyses were adjusted for covariates that may affect WL: age at diagnosis, sex, pre-diagnosis BMI, tumour type and stage.

Patients meeting the criteria for each of the proposed cachexia phenotypes were compared with patients who had lost <5% body weight as control.

To account for multiple testing, permutation testing was performed by running the adaptive permutation test in PLINK within each proposed phenotype. Permutation tests are often employed to adjust groups of correlated tests for multiple testing, since conventional methods such as Bonferroni correction are overly conservative when tests are correlated(400). The adaptive permutation test in PLINK gives up permuting SNPs that are clearly not going to be significant. This greatly speeds up the permutation procedure, as SNPs that are not significant will drop out quickly, making it possible to evaluate properly the significance of the handful of SNPs that require millions of permutations.

Finally, candidate genes (and the SNPs in the corresponding gene regions) were grouped based on known functional similarity according to gene ontology using AmiGO (Supplementary Table 2). The set-based test in PLINK was used to analyse association between grouped SNPs and cachexia = phenotypes. The set-based test selects the best set of SNPs whose mean of these single SNP statistics is significant

after permutation, which is particularly suited to large scale candidate gene studies(401). The empirical p-values of the set-based test were obtained by a permutation of 10 000 times of phenotype labels.

3.11.3 Transcriptomic study (Chapter 6)

Pearson correlation analysis was conducted to assess the linear relationship between the considered phenotypes independently (SMI or WL) with the expression of transcripts from select candidate genes. T-test analysis was conducted to compare how SMI (or WL) values differed for patients with high expression versus those with low expression for each of the candidate genes. The high and low expression groups for each gene transcript were determined by sorting patients by expression intensity and subsequently splitting the patients into three equally sized groups: those with high expression, mid- expression and low expression. The extremes of gene expression were compared while leaving out middle values. Comparison of the distal ends of gene expression maximizes the chance of detecting differences. Given the inherent variation in measures of gene expression, separation of the compared categories in this fashion also limits potential classification error. The cases considered for SMI and WL phenotypes for gene expression were based on sorting of transcript expression in all of the samples and binning based on extremes of gene expression as described above. The samples used for SNP studies (based on WL or muscularity) are non-matched as these two were independently designed studies. Transcriptome profiles were obtained from a single biopsy specimen along the disease trajectory of patients (static but not a dynamic read-out). Caution was exercised in over-interpreting the data with the emphasis to seek nominal relationships as it was not possible to delineate the expression from early vs. late response genes. A mechanistic understanding of the role of early and late response genes and their spatiotemporal relationships could be clearly discerned in animal models of cachexia (207), as described earlier.

CHAPTER 4

Clinical Classification of Cancer Cachexia: Phenotypic Correlates in Human Skeletal Muscle

4.1 Summary

4.1.1 Aims

One impediment towards the effective treatment of cachexia is a validated classification system. The aim of this Chapter was to investigate the changes in muscle fibre biology with regards to morphological structure and composition, to study alteration in various pathways that may account for altered fibre size and relate these changes to the different diagnostic criteria that have been proposed as part of the recent international consensus on the classification of cancer cachexia (11). The validated criteria could then be used to phenotype patients for gene – association studies (Chapter 6).

4.1.2 Methods

41 patients with resectable upper gastrointestinal (GI) or pancreatic cancer underwent characterisation for cachexia based on weight-loss (WL) and/or low muscularity (LM). Four diagnostic criteria were used >5%WL, >10%WL, LM, and LM+>2%WL. All patients underwent biopsy of the rectus muscle. Analysis included immunohistochemistry for fibre size and type, protein and nucleic acid concentration, Western blots for markers of autophagy, SMAD signalling, and inflammation.

4.1.3 Findings

Compared with non-cachectic cancer patients, patients with LM or LM+>2%WL, mean muscle fibre diameter was reduced by about 25% ($p=0.02$ and $p=0.001$ respectively). No significant difference in fibre diameter was observed if patients had WL alone. Regardless of classification, there was no difference in fibre number or proportion of fibre type across all myosin heavy chain isoforms. Mean muscle protein content was reduced and the ratio of RNA/DNA decreased in patients with either >5%WL or LM+>2%WL. Compared with non-cachectic patients, SMAD3 protein levels were increased in patients with >5%WL ($p=0.022$) and with >10%WL, beclin ($p=0.05$) and ATG5 ($p=0.01$) protein levels were increased. There were no differences in phospho-NFkB or phospho-STAT3 levels across any of the groups.

4.1.4 Conclusion

Muscle fibre size, biochemical composition and pathway phenotype can vary according to whether the diagnostic criteria for cachexia are based on weight loss alone, a measure of low muscularity alone or a combination of the two. For characterisation / biomarker studies (e.g. gene association) or intervention trials (where the primary end-point is a change in muscle mass or function), use of combined diagnostic criteria may allow identification of a more homogeneous patient cohort, reduce the sample size required and enhance the time scale within which trials can be conducted.

4.2 Introduction

Cancer cachexia has been defined recently 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 (11). Skeletal muscle loss appears to be the most significant event in cancer cachexia and is associated with a poor outcome (11, 12). The international consensus on the classification of cancer cachexia suggested that diagnostic criteria should take into account not only that weight loss is a signal event of the cachectic process but that the initial reserve of the patient should also be considered (either low BMI or low level of muscularity). Although the latter concept has some validation in terms of clinical risk (12), there has been no evaluation of the biological correlates in terms of changes within skeletal muscle itself.

Human skeletal muscle is composed of muscle fibres that are classified depending on their speed of contraction and predominant type of energy metabolism. Muscle fibres can be classified as type I (slow-twitch) and type II (fast-twitch) fibres based on their predominant myosin heavy chain (MyHC) isoform content. Generally, type I and type IIa fibres utilise oxidative phosphorylation, whereas type IIx and IIb fibres harness primarily anaerobic metabolism to generate ATP. Both the percentage and structural morphology of the fibre type will determine the phenotypic capacity and functional performance of any given muscle. Environmental factors in both health and disease have a direct impact leading to changes in fibre type / morphology and consequent functionality; such processes include aging, exercise, chronic disease, and cachexia

(232, 236-239). The change, preservation or loss of fibres may influence clinical symptoms and there is some evidence that all types of MyHC is targeted selectively in cancer cachexia (70). Ongoing loss of protein in muscle tissue may lead to muscle fibre shrinkage and a reduction in cross-sectional area (CSA). Equally, loss of muscle fibre CSA may lead to loss of aerobic capacity (VO_2 max) in healthy subjects as well as cancer patients (237, 245).

Although systemic inflammation is generally thought to be an important upstream mediator of cancer cachexia(18), the precise molecular mechanisms that mediate the changes in protein synthesis and degradation that ultimately lead to atrophy of muscle fibres in cancer cachexia in humans are not known. For each animal model that has been studied, different pathways have been implicated. From such animal models there is a predominant impression that increased degradation via activation of the ubiquitin proteasome pathway (UPP) is important (18). In contrast, human data is very limited. Activation of protein degradation via the UPP has not been a consistent finding (177) (402). This has led to suggestions that autophagy may be important or that pathways that may influence both synthesis and degradation may be important (e.g. TGF-β / SMAD signalling) (290).

In the present Chapter it was chosen to evaluate the relationship between the different cachexia definitions, systemic inflammation (serum C-reactive protein) and potential inflammatory signalling pathways within muscle (phospho-STAT3 and phospho-NFkB). Also examined was the potential for associations between the various cachexia definitions and activation of autophagy pathways or TGF-β / SMAD signalling.

The aim of this Chapter was to investigate the changes in muscle fibre biology with regards to morphological structure and composition, to study alteration in various pathways that may account for altered fibre size and relate these changes to the different diagnostic criteria that have been proposed as part of the recent international consensus on the classification of cancer cachexia (11). For characterisation / biomarker studies (e.g. gene association) or intervention trials (where the primary end-point is a change in muscle mass or function), use of combined, biologically validated diagnostic criteria may allow identification of a more homogeneous patient cohort, identify robust biomarkers e.g. gene association, and reduce the sample size required and enhance the time scale within which trials can be conducted.

4.3 Materials and methods

(Detailed methodology is found in Chapter 3)

4.3.1 Patient recruitment, identification, consent and ethics

Patients with resectable disease and suitable for the study were identified via the upper gastrointestinal cancer multi-disciplinary team (MDT) meetings at the Royal Infirmary, Edinburgh, UK. Written consent was given prior to entry into the study. All procedures were approved by the NHS Lothian local research ethics committee. The study conformed to the standards set by the Declaration of Helsinki (further details in Chapter 3).

4.3.2 Calculation of weight loss

Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. Although there may be recall bias, evidence to support the reliability of self-reported weight and weight history (403, 404) is well documented. Individual weight loss was calculated and expressed as percentage of pre-morbid body weight lost.

4.3.3 Classification of cancer cachexia

I) Weight loss >5% over past 6 months (in absence of simple starvation) (WL>5%)

II) Weight loss >10% over past 6 months (in absence of simple starvation) (WL>10%)

III) Stature adjusted skeletal muscle index consistent with low muscularity (LM) (see ‘CT-image analysis’ for cut-offs)

IV) Stature adjusted skeletal muscle index consistent with low muscularity and any degree of weight loss >2% (LM + >2%WL)

4.3.4 Rectus abdominis muscle biopsy

All biopsies were taken at the start of open abdominal surgery under general anaesthesia. The edge of the rectus abdominis was exposed and a 1cm^3 specimen removed using sharp dissection. The biopsy was cleaned of gross blood contamination. Obvious fat/fibrous tissue was removed prior to placement in a cryotube and being snap frozen in liquid nitrogen and stored at -80°C.

4.3.5 Rectus abdominis muscle sample preparation for cryo-section

A 0.1 – 0.5cm^3 section of muscle was cut. Liquid nitrogen was used to cool isopentane solvent in a tube to a temperature of ~-190°C. The section of muscle was stitched onto a segment of cork. OCT solution was placed at the junction between the cork base and the muscle. This was then lowered with the cork uppermost (i.e. muscle first) into cooled solvent and held for approximately 5 minutes (until the muscle was frozen). Samples were then stored at -80°C until use.

4.3.6 CT image analysis

CT scans used for analysis were performed solely for routine cancer care. The technique for CT image analysis has been described in detail in Chapter 3.

Estimates of whole body stores were generated from the raw data (cm^2) using the regression equations by Mourtzakis et al.(397), as detailed in Chapter 3

Cutoffs for low muscularity were based on a CT-based sarcopenic obesity study of cancer patients by Prado et al. (i.e., L3 skeletal muscle index: ≤38.5 cm^2/m^2 for women and ≤52.4 cm^2/m^2 for men) (28).

4.3.7 Immunohistochemistry

The frozen muscle sections were co-stained to distinguish each fibre type. The paraffin sections were stained for phospho-STAT3. Images of the entire tissue section were acquired using a VS120 slide scanner (Olympus Corporation, Tokyo, Japan).

The distribution of myosin heavy chain fibre types, the cross section area of the individual fibres in the section, and the phospho-STAT3 positive nuclei and staining density were analysed using the proprietary image analysis platform ASTORIA (Automated Stored Image Analysis) developed by Novartis/Preclinical Safety. Further details in Chapter 3.

4.3.8 Tissue Preparation for DNA, RNA and Protein extractions

Skeletal muscle tissue was minced and ground on dry ice. Aliquots were weighed using an analytical balance (Mettler Toledo) and stored at -80°C until use.

4.3.9 DNA and RNA extraction and linearity of the extraction method

DNA and RNA from human skeletal muscle tissue was extracted and purified with the automated Maxwell 16 system (Promega, Duebendorf, Switzerland). Aliquots of 4-8mg human skeletal muscle tissue were used for all subsequent DNA and RNA extractions. Further details in Chapter 3.

For DNA extraction, the Maxwell 16 LEV Blood DNA Kit (Promega) was used with a slightly adapted protocol compared with the manual's instructions (Chapter 3). For RNA extraction, the Maxwell 16 LEV simplyRNA Tissue Kit was used (Promega), following the manual's instructions (Chapter 3).

4.3.10 Protein extractions

Protein was extracted according to the methods in Chapter 3 and the samples were stored at -80°C until further use.

4.3.11 Western blots

Western blotting was performed in line with the methods set out in Chapter 3, then were analyzed densitometrically using ImageJ software version 1.45 (NIH, Bethesda,

MD, USA; http://rsbweb.nih.gov/ij). Band intensity of each sample was normalised to that of α-tubulin.

4.3.12 C - reactive protein (CRP)

Serum CRP concentration was measured with an automated immunoturbidimetric assay by clinical chemistry department, Royal infirmary Edinburgh, using blood collected from patients at the time of recruitment and before any therapeutic intervention.

4.3.13 Statistical analysis

Results are expressed as mean (± SEM). Comparisons between groups were performed using unpaired Student's t tests, whereas possible relationships were evaluated using Pearson's correlations. Results were considered significant if p values were less than 0.05. The program SPSS (version 20, SPSS, Chicago, IL, USA) was used for all the statistical tests.

4.4 Results

4.4.1 Patient demographics

A total of 41 cancer patients with resectable UGI or pancreatic cancer were recruited. In general, patients were over 65 years of age, predominantly male and had sustained, on average, 5% loss of weight compared with pre-illness levels (Table 4.1). Patients were grouped based upon the concepts of the International Classification Framework (11) according to weight loss or weight loss in association with low muscularity. The specific phenotypes considered were weight loss >5% (WL>5%), weight loss >10% (WL>10%), low muscularity (LM), and LM with weight loss >2% (LM+>2% WL). Although BMI was reduced in all groups classified as cachectic, only the LM and LM+>2% WL groups had a significantly lower fat free mass index (Table 4.1).

	All Patients (n = 41)	Weight Stable (n = 23)	Weight Loss >5% (n = 18)	p	Weight Stable (n = 30)	Weight Loss >10% (n = 11)	p	Normal Muscularity (n = 9)	Low Muscularity (n = 32)	p	Not Low Muscularity + >2% W/L (n = 24)	Low Muscularity + >2% W/L (n = 17)	p
Age	65 ± 2	67 ± 2	63 ± 2	0.216	65 ± 2	66 ± 2	0.849	61 ± 3	66 ± 2	0.128	65 ± 2	66 ± 2	0.727
Sex (M:F)	30 : 11	19 : 4	11 : 7		23 : 7	7 : 4		4 : 5	26 : 6		19 : 5	11 : 6	
Pre Illness Weight (kg)	83 ± 3	83 ± 4	81 ± 4	0.748	82 ± 3	83 ± 5	0.912	86 ± 7	81 ± 3	0.487	84 ± 4	81 ± 4	0.602
Weight Loss (%)	5 ± 1	0 ± 1	12 ± 1	**0.000***	2 ± 1	15 ± 2	**0.000***	5 ± 3	6 ± 1	0.752	2 ± 1	11 ± 1	**0.000***
BMI	26 ± 1	28 ± 1	24 ± 1	**0.016***	27 ± 1	24 ± 1	**0.048***	29 ± 2	25 ± 1	**0.050***	28 ± 1	24 ± 1	**0.013***
Body Fat (kg)	17 ± 1	18 ± 2	16 ± 1	0.416	17 ± 1	16 ± 1	0.609	16 ± 3	17 ± 1	0.768	18 ± 2	16 ± 1	0.258
Fat Free Mass Index (FFMI) (kg / m^2)	16 ± 0	16 ± 1	15 ± 1	0.057	16 ± 1	15 ± 1	0.143	17 ± 1	15 ± 0	**0.026***	17 ± 1	14 ± 0	**0.001***
CRP (mg/L)	14.5 ± 5	16 ± 8	12 ± 5	0.667	13 ± 6	18 ± 8	0.670	15 ± 9	14 ± 6	0.966	10 ± 4	20 ± 10	0.301

Table 4.1 - Demographic data of the patients involved in the study. Data (except gender split) are presented as mean (SEM). * = cachexia group significantly different from the non-cachexia group, ($p < 0.05$ by Student's t test). Abbreviations – CRP = C - reactive protein, BMI = Body Mass Index.

4.4.2 Muscle fibre size, number, and type

If patients were classified as cachectic by LM or LM + >2%WL, fibre size was reduced significantly (all types of myosin heavy chain fibre) when compared with non-cachectic patients and controls (Figure 4.1A). The association of cachexia with reduced fibre size was not observed if patients were classified according to WL alone. Representative immunohistological sections demonstrating differences in fibre diameter between a healthy control and an individual in Group II versus Group IV is shown in Figure 4.1B. Immunohistology for type I and IIa resulted in complementary staining in general, whereas fibre type IIb resulted in very low staining intensity as reported elsewhere (405); therefore quantitative analysis was done only with type I and IIa but not with type IIb (Table 4.2). As would be expected from a decrease in fibre size, there was a trend across all groups for fibre density to increase in those with cachexia. However, due to large variability, this was not statistically significant. There was no evidence of selective fibre atrophy across any of the classification groups (Table 4.2).

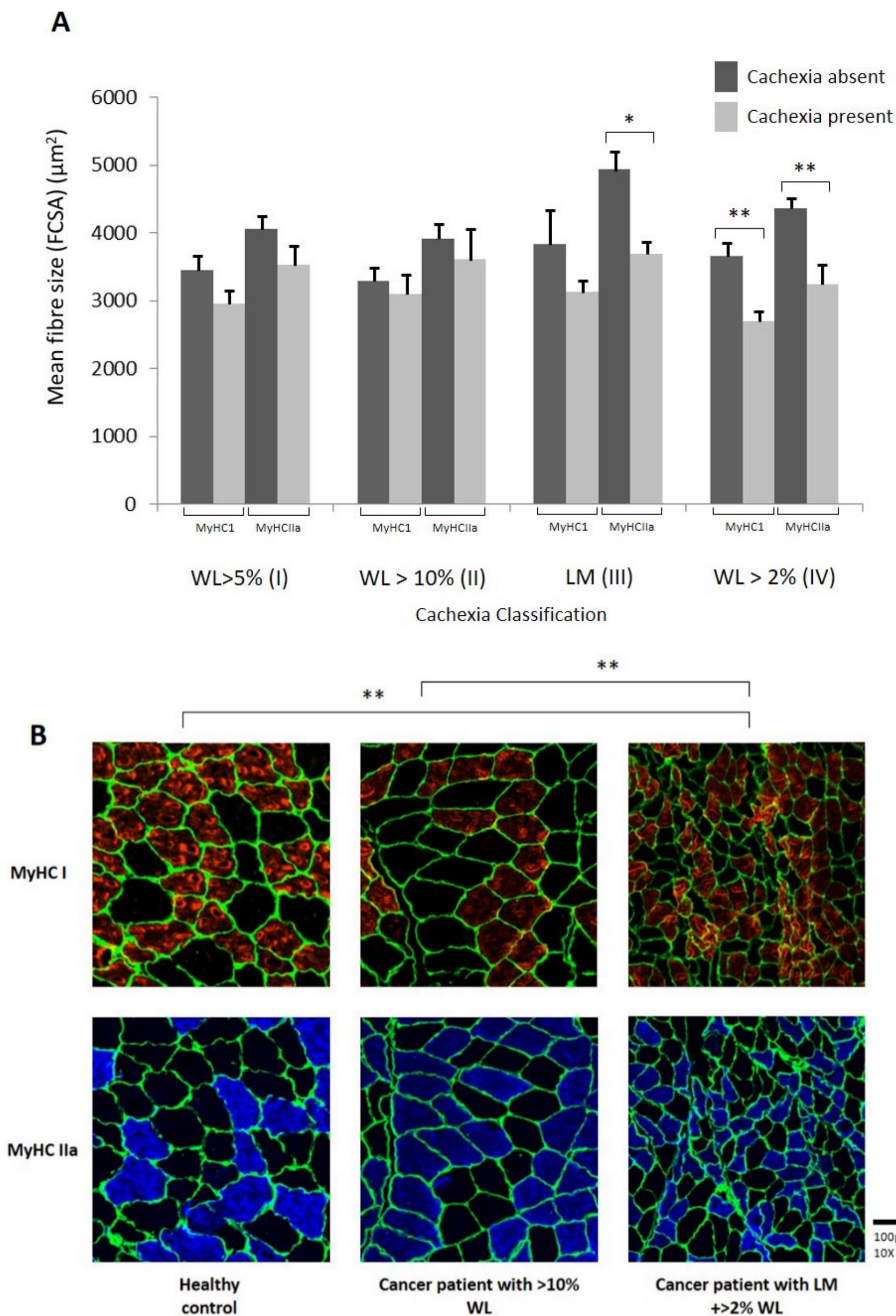


Figure 4.1 - Fibre type cross sectional area (FCSA) according to different definitions of cachexia. (A) Mean (± SEM) fibre size for both MyHC1 and MyHCIIa. A comparison is made between patients with the proposed cachexia definition absent (dark grey) and those with the proposed cachexia definition present (light grey) for the four definitions set out in Methods (I-IV). (*, P < 0.05 and **, P < 0.01, by Student's t test). (B) Immunohistological sections of muscle for a healthy control, patient with weight loss alone (10.1%) (Group II), and patient with low muscularity and >2% weight loss (Group IV). Laminin is shown in green, MyHC1 shown in red, and MyHCIIa is shown in blue.

	All Patients (n = 27)	Weight Stable (n = 15)	Weight Loss >5% (n = 12)	p-Value	Weight Stable (n = 19)	Weight Loss >10% (n = 8)	p-Value	Normal Muscularity (n = 4)	Low Muscularity (n = 23)	p-Value	Not Low Muscularity + >2% W/L (n = 15)	Low Muscularity + >2% W/L (n = 12)	p-Value
Age	66 ± 2	67 ± 2	64 ± 2	0.321	66 ± 2	65 ± 2	0.791	63 ± 3	66 ± 2	0.370	65 ± 2	66 ± 2	0.882
Sex (M:F)	24 : 3	15 : 0	9 : 3		18 : 1	6 : 2		4 : 0	20 : 3		15 : 0	9 : 3	
Pre Illness Weight (kg)	85 ± 4	83 ± 5	88 ± 5	0.537	83 ± 4	89 ± 6	0.482	104 ± 12	82 ± 3	**0.018***	86 ± 5	84 ± 5	0.737
Weight Loss (%)	5 ± 1	0 ± 1	12 ± 1	**0.000***	2 ± 1	14 ± 2	**0.000***	5 ± 2	5 ± 2	0.947	1 ± 1	11 ± 2	**0.000***
BMI at time of biopsy	26 ± 1	27 ± 2	25 ± 1	0.381	29 ± 1	25 ± 1	0.454	33 ± 3	25 ± 1	**0.003***	28 ± 1	24 ± 1	**0.035***
Body Fat (kg)	25 ± 1	18 ± 2	18 ± 1	0.980	18 ± 1	17 ± 1	0.848	22 ± 3	17 ± 1	0.103	19 ± 2	16 ± 1	0.255
Free Fat Mass (FFM) (kg)	48 ± 2	49 ± 2	46 ± 2	0.217	48 ± 2	46 ± 2	0.459	59 ± 3	46 ± 1	**0.000***	51 ± 2	44 ± 2	**0.012***
Free Fat Mass Index (FFM) (kg / m^2)	27 ± 0	16 ± 1	15 ± 1	0.303	16 ± 1	15 ± 1	0.511	19 ± 1	15 ± 0	**0.000***	17 ± 1	14 ± 0	**0.005***

Table 4.2 - Continued over page

	All Patients (n = 27)	Weight Stable (n = 15)	Weight Loss >5% (n = 12)	p-Value	Weight Stable (n = 19)	Weight Loss >10% (n = 8)	p-Value	Normal Muscularity (n = 4)	Low Muscularity (n = 23)	p-Value	Not Low Muscularity + >2% W/L (n = 15)	Low Muscularity + >2% W/L (n = 12)	p-Value
Mean fibre size (FCSA) (μm^2)													
MyHC All Data	3588 ± 172	3800 ± 190	3324 ± 297	0.167	3639 ± 180	3468 ± 412	0.514	4509 ± 382	3408 ± 172	**0.020***	4045 ± 169	2979 ± 234	**0.001***
Group MyHCI	3232 ± 169	3446 ± 241	2963 ± 225	0.183	3287 ± 206	3101 ± 319	0.621	3832 ± 616	3127 ± 167	0.145	3653 ± 229	2705 ± 159	**0.004***
Group MyHCIIa	3831 ± 195	4076 ± 195	3524 ± 361	0.167	3924 ± 201	3611 ± 478	0.482	4934 ± 290	3698 ± 193	**0.016***	4365 ± 164	3238 ± 297	**0.002***
Total fibre number per mm^2													
MyHC All Data	1228 ± 147	1007 ± 224	1504 ± 187	0.152	1018 ± 184	1726 ± 252	0.119	937 ± 179	1296 ± 169	0.398	1226 ± 227	1263 ± 183	0.905
Group MyHCI	627 ± 96	525 ± 160	756 ± 92	0.427	506 ± 127	915 ± 133	0.217	406 ± 86	666 ± 112	0.353	653 ± 160	596 ± 97	0.777
Group MyHCIIa	621 ± 94	503 ± 147	769 ± 101	0.067	519 ± 120	863 ± 131	0.163	594 ± 117	693 ± 106	0.706	682 ± 146	676 ± 142	0.976
Fibre Type (%)													
MyHCI	48 ± 3	49 ± 4	41 ± 3	0.128	48 ± 3	38 ± 5	0.079	43 ± 3	46 ± 3	0.691	48 ± 4	42 ± 4	0.230
MyHCIIa	55 ± 2	52 ± 3	59 ± 2	0.107	54 ± 3	58 ± 3	0.388	64 ± 3	53 ± 2	0.099	55 ± 3	55 ± 3	0.978

Table 4.2 - Myosin heavy chain fibre data of the patients involved in the study. Data (except gender split) are presented as mean (SEM). * = cachexia group significantly different from the non-cachexia group, ($p < 0.05$ by Student's t test). Abbreviations – BMI = Body Mass Index, FCSA = Fibre Cross Sectional Area.

4.4.3 Protein content

The results for skeletal muscle protein content are shown in Figure 4.2A. When compared with non-cachectic patients, muscle protein content was reduced significantly (approximately 13%) in patients with either >5% WL or LM + >2%WL (Figure 4.2A and Table 4.3). However if the LM criteria were applied alone no difference in the protein content was observed. In addition, patients with >10% WL showed a 10% reduction in protein content when compared with non-cachectic patients but this difference did not reach statistical significance (Figure 4.2A and Table 4.3).

4.4.4 RNA, DNA, and RNA / DNA Ratio

The results for skeletal muscle DNA and RNA content are also shown in Figure 4.2(B,C, and D) and Table 4.3. RNA content was not significantly different in cachectic patients when compared with non-cachectic patients according to any of the diagnostic criteria (Figure 4.2B and Table 4.3). In contrast, DNA content was increased by 50% with >5% WL but decreased by ~40% in patients with LM (Figure 4.2C). The ratio of RNA / DNA was decreased (approximately 30%) in patients with >5% WL and LM + >2%WL (Figure 4.2D).

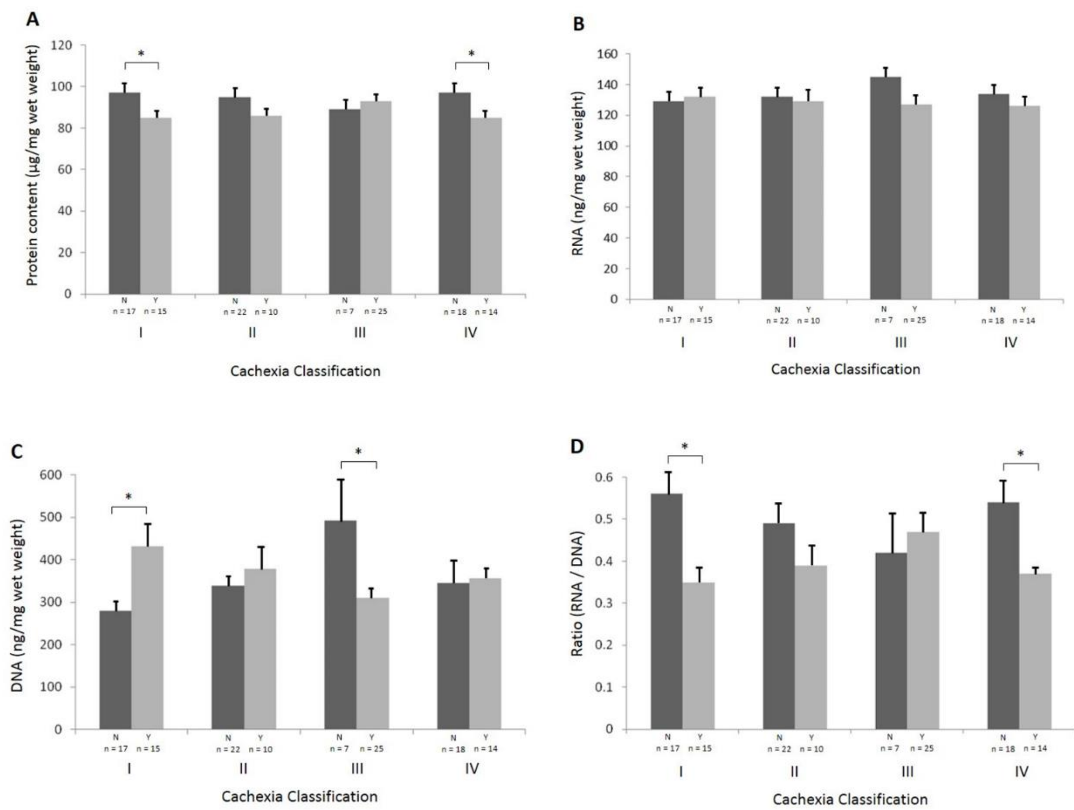


Figure 4.2 - Variations in protein and nucleic acid content according to the different definitions of cancer cachexia. A comparison is made between patients with the proposed cachexia definition absent (dark grey) and those with the proposed cachexia definition present (light grey) for the four definitions set out in the methods (I-IV). (A) Mean (± SEM) wet weight protein content. (B) Mean (± SEM) RNA content. (C) Mean (± SEM) DNA content. (C) Mean (± SEM) RNA / DNA ratio. (*, P < 0.05 by Student's t test).

	All Patients (n = 32)	Weight Stable (n = 17)	Weight Loss >5% (n = 15)	p-Value	Weight Stable (n = 22)	Weight Loss >10% (n = 10)	p-Value	Normal Muscularity (n = 7)	Low Muscularity (n = 25)	p-Value	Not Low Muscularity + > 2% W/L (n = 18)	Low Muscularity + > 2% W/L (n = 14)	p-Value
RNA (ng/mg wet weight)	131 ± 6	129 ± 8	132 ± 9	0.859	132 ± 7	129 ± 13	0.847	145 ± 9	127 ± 7	0.220	134 ± 8	126 ± 10	0.520
DNA (ng/mg wet weight)	351 ± 34	279 ± 33	431 ± 54	**0.019***	338 ± 38	378 ± 66	0.582	493 ± 103	310 ± 28	**0.020***	345 ± 53	357 ± 34	0.866
Ratio (RNA / DNA)	0.46 ± 0.05	0.56 ± 0.07	0.35 ± 0.04	**0.013***	0.49 ± 0.06	0.39 ± 0.05	0.254	0.42 ± 0.11	0.47 ± 0.05	0.622	0.54 ± 0.07	0.37 ± 0.02	**0.050***
Protein content (µg/mg wet weight)	92 ± 2.7	97 ± 4	85 ± 2	**0.015***	95 ± 4	86 ± 3	0.132	89 ± 4	93 ± 3	0.575	97 ± 4	85 ± 2	**0.035***
Protein content / RNA	0.77 ± 0.05	0.83 ± 0.09	0.71 ± 0.07	0.273	0.79 ± 0.07	0.75 ± 0.09	0.742	0.63 ± 0.05	0.82 ± 0.07	0.154	0.78 ± 0.08	0.76 ± 0.07	0.827

Table 4.3 - Protein, DNA, and RNA content of the patients involved in the study. Data (except gender split) are presented as mean (SEM). * = cachexia group significantly different from the non-cachexia group, ($p < 0.05$ by Student's t test).

4.4.5 Autophagy pathways

All raw Western blotting data can be seen in Figure 4.6. In patients with >10% WL, Beclin and ATG5 protein levels were increased significantly in cachectic patients when compared with non-cachectic patients (Figure 4.3). ATG7 and 12 levels were not different in cachectic patients when compared with non – cachectic patients according to any of the diagnostic criteria (Table 4.4).

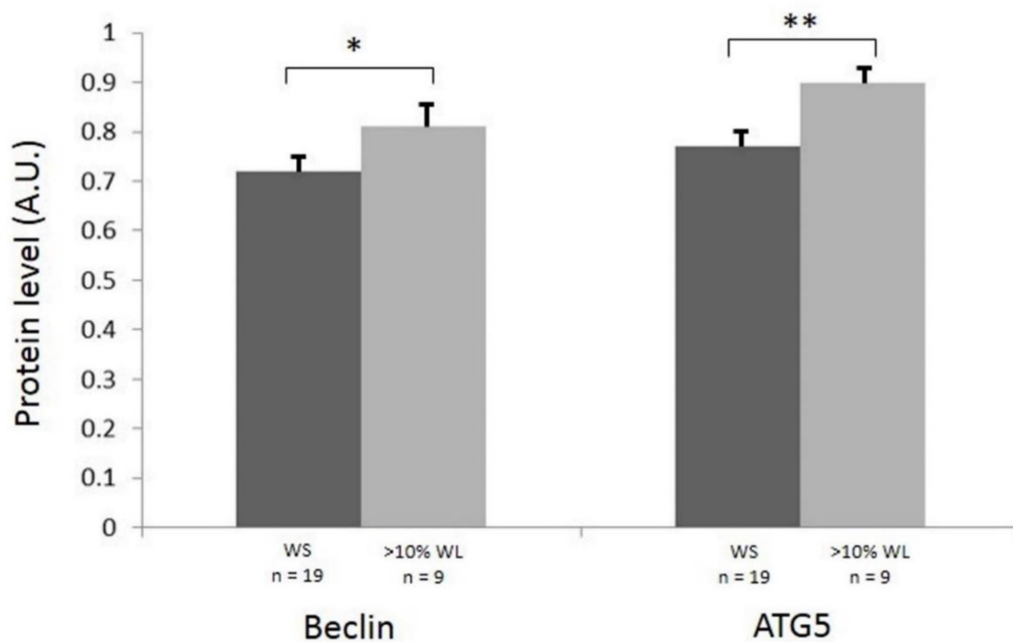


Figure 4.3 - Skeletal muscle Beclin and ATG5 protein levels in patients with or without >10% weight loss (Group II). Western blot analysis with indicated antibodies, α-tubulin was used as a loading control. Graph shows the mean (± SEM) protein level represented in arbitrary units (A.U). (*, $P < 0.05$ and **, $P < 0.01$, by Student's t test).

	Weight Stable (n = 14)	Weight Loss >5% (n = 14)	p-Value	Weight Stable (n = 19)	Weight Loss >10% (n = 9)	p-Value	Normal Muscularity (n = 8)	Low Muscularity (n = 20)	p-Value	Not Low Muscularity + >2% W/L (n = 17)	Low Muscularity + >2% W/L (n = 11)	p-Value
Beclin (A.U)	0.73 ± 0.03	0.77 ± 0.04	0.452	0.72 ± 0.02	0.81 ± 0.05	**0.050***	0.70 ± 0.03	0.77 ± 0.03	0.232	0.72 ± 0.03	0.79 ± 0.04	0.153
ATG5 (A.U)	0.80 ± 0.03	0.83 ± 0.04	0.614	0.77 ± 0.03	0.9 ± 0.04	**0.013***	0.75 ± 0.05	0.84 ± 0.03	0.137	0.81 ± 0.03	0.82 ± 0.05	0.865
ATG12 (A.U)	0.86 ± 0.04	0.87 ± 0.04	0.898	0.84 ± 0.03	0.93 ± 0.05	0.090	0.83 ± 0.05	0.89 ± 0.03	0.313	0.86 ± 0.03	0.88 ± 0.04	0.720
ATG7 (A.U)	0.89 ± 0.05	0.87 ± 0.05	0.741	0.85 ± 0.04	0.96 ± 0.07	0.136	0.85 ± 0.08	0.89 ± 0.04	0.617	0.89 ± 0.04	0.86 ± 0.06	0.682
phospho-SMAD3 (A.U)	0.88 ± 0.04	0.98 ± 0.04	0.116	0.91 ± 0.04	0.97 ± 0.04	0.343	0.87 ± 0.07	0.95 ± 0.03	0.171	0.90 ± 0.04	0.98 ± 0.04	0.191
SMAD3 (A.U)	0.82 ± 0.03	0.92 ± 0.03	**0.022***	0.86 ± 0.02	0.89 ± 0.04	0.497	0.88 ± 0.03	0.87 ± 0.03	0.816	0.85 ± 0.02	0.89 ± 0.04	0.348
phospho-SMAD3/SMAD3 (A.U)	1.08 ± 0.06	1.08 ± 0.05	0.972	1.07 ± 0.05	1.11 ± 0.07	0.630	0.98 ± 0.05	1.12 ± 0.05	0.107	1.06 ± 0.05	1.12 ± 0.06	0.467
phospho-NF-κB (A.U)	0.97 ± 0.03	1.00 ± 0.04	0.608	0.96 ± 0.02	1.03 ± 0.05	0.116	0.94 ± 0.03	1.00 ± 0.03	0.258	0.97 ± 0.02	1.01 ± 0.04	0.347
phospho-STAT3 staining density of nuclei (A.U.)	17.9 ± 1.34	15.5 ± 0.62	0.105	17.4 ± 1.03	15.1 ± 0.74	0.170	16.3 ± 0.95	16.7 ± 1.00	0.817	17.2 ± 1.10	15.6 ± 0.80	0.311

Table 4.4 - Western blots for protein markers of cellular signalling. Data are presented as mean (SEM). Immunohistology data is shown for phospho-STAT3 and is recorded as staining density of nuclei (A.U.). * = cachexia group significantly different from the non-cachexia group, ($p < 0.05$ by Student's t test). Abbreviations – A.U = arbitrary units.

4.4.6 SMAD signalling

In patients with >5% WL, SMAD3 protein levels were significantly increased when compared with non-cachectic patients (Figure 4.4). There were no significant differences in phospho-SMAD3 / SMAD3 across any of the groups (Figure 4.4).

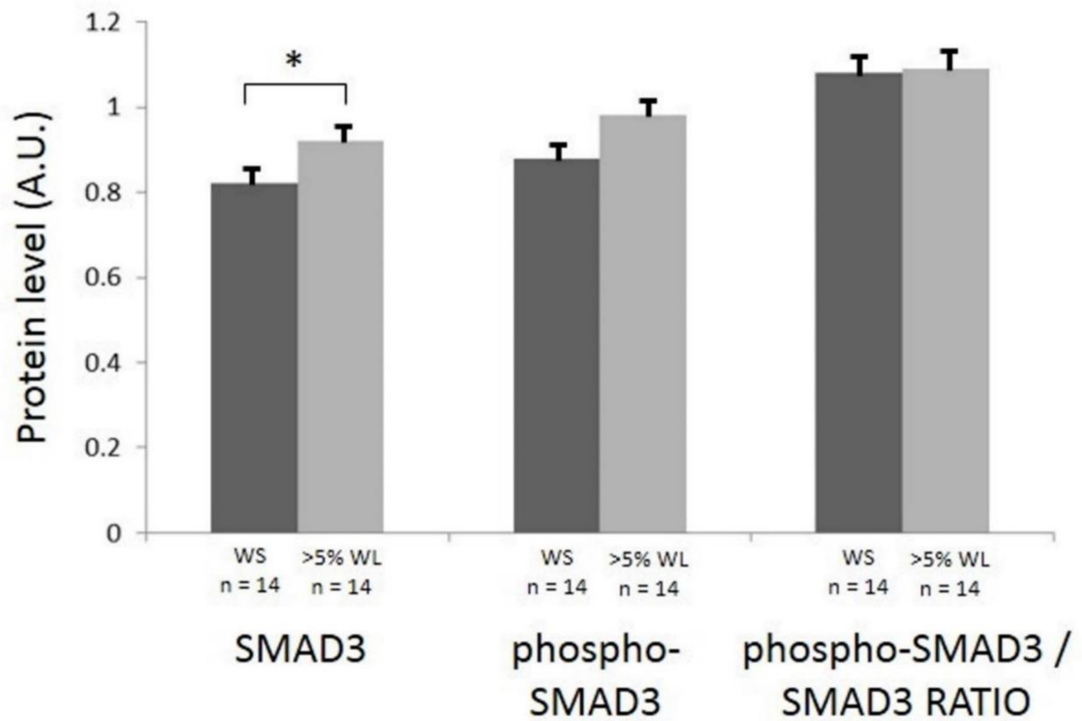


Figure 4.4 - Total SMAD3, phospho-SMAD3 and ratio of phospho-SMAD3 / SMAD3 in patients with or without >5% WL (Group I) levels. Western blot analysis with indicated antibodies, α-tubulin was used as a loading control. Graph shows the mean (± SEM) protein level represented in arbitrary units (A.U). (*, P < 0.05 by Student's t test).

4.4.7 Inflammatory pathways

Systemic inflammation was estimated using patients' serum CRP levels (Table 4.1). Patients were classified as having systemic inflammation if their CRP was ≥ 10 mg/L. There was no difference in the proportion of patients with or without systemic inflammation according to the definition of cachexia. Levels of phospho-NFκB and phospho-STAT3 were not significantly different in patients with or without cachexia (using any of the definitions: Table 4.4) or with or without systemic inflammation (Figure 4.5).

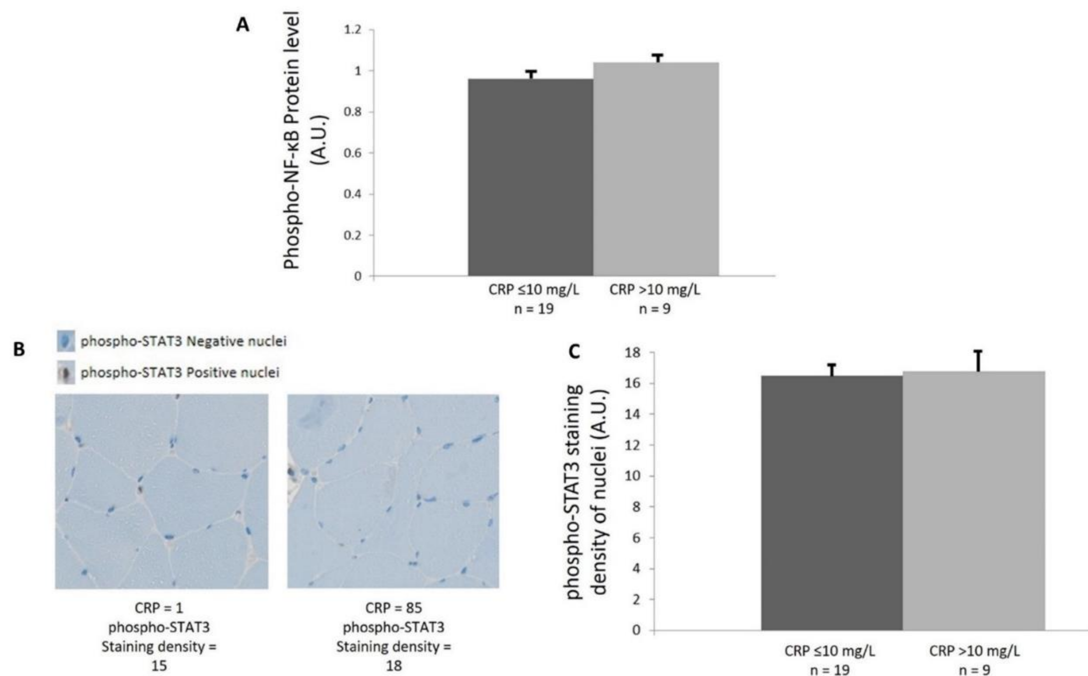


Figure 4.5 - Inflammatory pathways in patients with (CRP >10mg/L) and without (CRP ≤10mg/L) systemic inflammation. (A) Graph shows the mean (± SEM) protein level of phospho-NF-κB, represented in arbitrary units (A.U). (B) Representative immunohistochemistry and nuclei count of phospho-STAT3 (area shown is representative of field) of a patient with or without systemic inflammation. (C) Graph shows the staining density of phospho-STAT3 nuclei (A.U.) (± SEM) in the presence or absence of systemic inflammation.

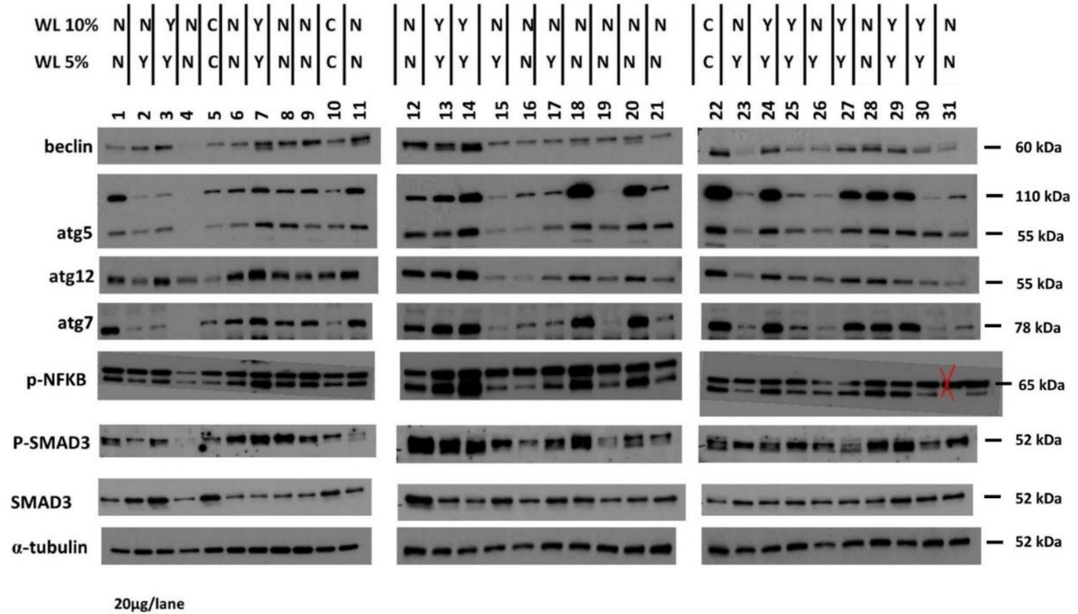


Figure 4.6 - Western Blots (beclin, atg5, atg7, atg12, p-NFKB, P-SMAD3, SMAD3, and alpha tubulin) of protein extracts from the skeletal muscle of healthy controls (C) and cancer patients with (Y: yes) or without (N: no) weight-loss (WL) >5% or >10% of pre-illness stable weight. Each gel for each protein was run separately. Alpha-tubulin (loading control) was not included in each gel but rather was used as a benchmark for the standardised 20 micro gram of protein added to every well of each gel. The blot for NFKB for patient 31 is moved one to the right as the initial lane was loaded incorrectly.

4.5 Discussion

4.5.1 Fibre size

The diagnostic criterion for cancer cachexia has long been based on weight loss alone (11) and can reflect loss in either fat or lean tissue compartments. Given that the key tissue loss in cancer cachexia is considered to be skeletal muscle, a recent consensus process suggested that the diagnostic criteria for cachexia should also take account of low baseline levels of muscularity (11). In the present study, when patients were classified as cachectic or not according to ≥ 5% weight loss there was no significant difference in whole body muscularity (FFMI) or muscle fibre CSA. In contrast, when patients were classified according to low muscularity and ≥ 2% weight loss, FFMI was decreased and fibre cross sectional area was also significantly reduced (Figure 4.1). Such findings demonstrate that heterogeneity in relation to low muscularity and fibre atrophy may be reduced according to the clinical definition of cachexia. This finding may be important especially when considering inclusion criteria for clinical trials that aim to test the efficacy of drugs targeted at reversal of muscle wasting in cancer patients. The reduction in fibre size in all MyHC isoforms observed in the present study is consistent with previous animal (254) and human studies of cancer cachexia (236-239). The rectus muscle of patients with oesophago-gastric cancer cachexia has been shown to lose all type MyHC content as well as undergo a reduction in fibre size (236). Equally, in pancreatic cancer patients with cachexia, both type I and type II MyHC protein levels were decreased by 45% when compared with controls (238).

4.5.2 Fibre type

In order to study differences in muscle fibre morphology and composition within the different cachexia categories, immunohistochemical analysis of human muscle samples was performed. For that, the staining methods for the myosin heavy chain antibodies specific for the different fibre types (I, IIa, and IIb) was established and validated. Staining for type I and IIa fibres resulted in strong specific staining specificity, however only weak staining was observed against type IIb MyHC, this finding has also been reported elsewhere (405). The predominant types of MyHC fibre in rectus abdominis muscle are I and IIa and only <8% of type IIb positive fibres have previously been described (406). Of the adult skeletal isoforms, each are expressed to varying degrees in both mouse and human skeletal muscle. However, although MyHCIIb is highly expressed at both the messenger RNA (mRNA) and protein level

in murine skeletal muscle, evidence to date suggests that this isoform is effectively only expressed at the mRNA level in a very small subset of specialised muscles in the adult human (407). As mentioned above, MyHCIIb expression is typically associated with high forces of contraction combined with rapid contractile characteristics and it has been suggested that the contractile characteristics of MyHCIIb may be incompatible with the biomechanical constraints of larger muscles (408), which may account for the lack of specificity found in the rectus muscle of our patient population.

In cachexia there is conflicting evidence as to whether there is selective loss of fibre type. There was no evidence for selective loss of fibre type in the present study (Table 4.2). Evidence from animal models suggests that Type II fibres are targeted selectively (179), with relative preservation of type I fibres in fasting (249), exposure to glucocorticoids (250), sepsis (252) and in the gastrocnemius muscle of the C26 model of cancer cachexia (253, 327). Models of cardiac cachexia, however have suggested a trend to selective loss of type I fibres and an increase in type II fibre (240). Furthermore, not all groups have demonstrated Type I and II fibre differences even in animals. Indeed in a recent study of the C26 cachectic mouse model, both glycolytic and oxidative fibres of (extensor digitorum longus) EDL muscle underwent wasting (254), whilst in a previous study using the same mouse model there was a significant increase in the amount of MyHCIIb and a significant decrease in the amount of type 1 MyHC in soleus muscle (255). It is currently not entirely clear which type of fibres are affected in human cancer cachexia, however, in patients with oesophago-gastric cancer cachexia early loss of all MyHC isoforms has been reported (236).

The activity patterns of a muscle are also key in determining phenotype. If muscle cells are recruited infrequently they develop into fast/glycolytic units whereas if they are recruited more often, they form slow/oxidative units. In the C26 mouse model of cancer cachexia, there have been reports of switching of myosin isoforms in the soleus muscle of cachectic mice (255). In pancreatic cancer patients with cachexia, no difference in the ratio of fast/slow myosin isoform was demonstrated compared with controls (238).

4.5.3 Muscle RNA, DNA, and protein content

In the present study, when compared with non-cachectic patients, muscle protein content was reduced significantly (approximately 13%) if patients were classified as cachectic by either >5% WL ($p=0.015$) or LM + >2%WL ($p=0.035$), and by 10% in patients with >10% WL. Protein content expressed in relation to wet weight of muscle has been shown to decrease progressively (in excess of 50%) in the gastrocnemius muscle of mice bearing the MAC-16 tumour (257). This suggests that not only is there loss of fibre diameter, but that the quality of the fibre is altered with loss of either sarcoplasmic or myofibrillar protein. Such changes in fibre composition may contribute to the reduced muscle mechanical quality (force per unit cross-sectional area) observed in human cancer cachexia (213).

A reduction in both RNA content and activity in skeletal muscle has been attributed to a depression of protein synthesis in mice bearing the MAC16 tumour (257) . In the present study, RNA content was unaltered in cachectic patients (classified either with >5% WL or LM + >2% WL) compared with non-cachectic patients. A reduction in the RNA content in the muscle of mice bearing the Ehrlich ascites tumour has also been reported, but this occurred later than the observed depression in the rate of protein synthesis (258). Whether muscle protein synthesis is depressed in human cancer cachexia remains to be resolved (18).

In a murine model of cancer cachexia DNA content of the gastrocnemius muscle has been shown to remain relatively constant, despite the finding of a decrease in protein and RNA content (257). The current study demonstrated DNA content was increased by >50% with >5% WL but decreased by 40% in patients with LM (Figure 4.2C). Because mature myofibre nuclei are thought to be mitotically inactive, increased DNA content in skeletal muscle cells suggests activation of satellite cells (409) or infiltration by other cell types such as inflammatory cells or adipocytes. In the LM group, the decrease in DNA may be due to pre-existing age-related sarcopenia or other causes of muscle atrophy (e.g. immobilisation) and may relate to muscle specific apoptosis and reduction in cell number in keeping with a reduction in muscle mass on CT scanning. The diametrically opposite changes in muscle DNA content dependent on whether patients are classified according to weight-loss or low muscularity again underpin the potential diverse mechanisms whereby older cancer patients may develop a low level of muscularity.

The issue of whether nuclear domain size is reduced in cancer cachexia remains to be resolved. In particular, whether apoptosis in skeletal muscle is increased in cancer cachexia and the degree to which DNA content is maintained or not via a compensatory increase in myonuclear number (possibly via satellite cell nuclei incorporation) is not known. Features of cachexia such as hypogonadism (resulting in low testosterone) or systemic inflammation (associated with high IL-6) could influence such regenerative capacity. In the current study RNA / DNA was altered in the cachectic patients (independent of definition) compared with the non- cachectic patients. This may be due to the interplay of the mechanisms described above.

4.5.4 Mechanisms

Skeletal muscle atrophy may occur as a result of decreased synthesis, increased degradation or both (79). In mice bearing the MAC-16 adenocarcinoma, muscle loss is due to the combination of reduced synthesis and increased degradation (277). Similarly Samuels et al demonstrated reduced protein synthesis and increased degradation in skeletal muscle co-incident with the onset of cachexia in mice implanted with the C26 murine model (410). Recently a study used a novel technique to measure habitual myofibrillar protein synthesis in patients with cancer compared with healthy controls. Contrary to previous studies, there was no evidence of suppression of myofibrillar protein synthesis in patients with cancer cachexia. This finding implies a small increase in muscle breakdown may account for muscle wasting (80).

4.5.5 Degradation pathways

The majority of signalling pathways contributing to muscle atrophy in pre-clinical models are mediated through activation of the ubiquitin-proteasome proteolytic pathway (UPP) (82). The muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1 are up regulated in animal models of acute atrophy (83, 84), and MuRF1 selectively targets the myofibrillar protein myosin heavy chain resulting in muscle wasting (70). However, the role of the E3 ligases in human cachexia is less well defined. The current study did not measure directly these pathways as results from our previous investigation on a similar cohort of patients found no up regulation using a transcriptomics approach (89), this has also been validated recently in a

separate cohort of patients with gastric cancer(411). In the present study autophagy proteins (ATG) 5, 7, 12, and beclin 1 were studied. These proteins are necessary for autophagy due to their role in autophagosome elongation (412). When patients were classified according to >10% WL, Beclin and ATG5 protein levels were significantly increased in cachectic patients when compared with non-cachectic patients. In a previous study in a similar cohort of patients, the autophagy related genes GABRAPL1 and BNIP3 were increased in rectus muscle biopsies from cachectic versus non-cachectic patients (89). In normal muscle, low-protein diets up-regulate autophagy that leads to the loss of muscle mass at least partially through lysosomal degradation (413). Intriguingly, under other circumstances decreased autophagy can also lead to muscle atrophy.

4.5.6 Systemic Inflammation

Systemic inflammation is thought to be a major mediator of cancer cachexia (18). However, the relationship between inflammation in the systemic compartment versus muscle and the relationship of either to muscle loss in humans is not clear. In the systemic compartment, Il-6 is thought to be a major mediator and may signal within target organs via STAT-3. Alternatively, both IL-1 and TNF alpha may signal via NF-kB. NF-kB regulation of muscle atrophy is predominantly executed by promoting proteasome-mediated degradation (143). Activation of NF-kB has been detected in both physiological and pathological atrophic conditions such as denervation, unloading, aging, cancer, sepsis, diabetes, and such atrophy can be reversed by pharmacologic or genetic NF-kB inhibition (414). In the present study although there was evidence for systemic inflammation in a proportion of patients, no significant difference was found in the levels of phospho-NFkB or phospho-STAT3 across any of the definitions of cachexia or in those with or without evidence of systemic inflammation. It is possible that inflammatory mediators have their main effects on muscle atrophy via central mechanisms mediated via the CNS (33).

4.5.7 SMAD3

It has been suggested that binding of myostatin to the ACVR2B receptor results in the phosphorylation of two serine residues of SMAD2 or SMAD3. This leads to the assembly of SMAD2/3 with SMAD4 to the heterodimer that is able to translocate to

the nucleus and activate transcription of target genes (415). One of the known downstream targets of SMAD signalling is MyoD, a transcriptional factor that is involved in skeletal muscle development and takes part in the repair of damaged skeletal muscle (416). Moreover, SMAD signalling targets other genes such as myf5 and myogenin, known to be important for myogenesis (417). Myostatin is upregulated in cachexia and in states of muscle paralysis (418). Myostatin/ACVR2B activates SMAD2/3 signalling and importantly SMAD2/3 inhibition completely desensitises ACVR2B-induced muscle atrophy (290). Inhibition of myostatin by a dominant negative ACVR2B promotes muscle hypertrophy independent of muscle satellite cell recruitment consistent with a direct signalling effect on muscle catabolism (290). When patients were classified as cachectic according to >5% WL, SMAD3 protein levels were significantly increased in cachectic patients when compared with non-cachectic patients. Equally there was a similar (but not significant) increase in phospho-SMAD3 associated with >5% weight loss. It is not known whether such increased protein levels indicate increased pathway activity independent of any alteration in the ratio of phospho-SMAD3/SMAD3.

4.5.8 Limitations of study

It is important to appreciate that the majority of patients in the present series will have had some degree of age-related sarcopenia, that this will necessarily co-exist with any cancer specific loss of skeletal muscle mass and that the diagnostic criteria used in the present study will not necessarily separate one from the other. The current study was not longitudinal and it was therefore not possible to document active muscle loss. It is also important to recognise that when patients were divided into different diagnostic categories the sample size in individual categories may have limited the ability to detect a statistical difference or not. This was an exploratory study and provides the basis for a larger study with adequate statistical power for definitive analysis.

4.5.9 Conclusions

In the present Chapter, when the diagnostic criteria for cachexia included both a measure of low muscularity and weight loss, muscle fibre size, protein content and RNA/DNA content were all reduced. Such consistent findings were not observed when cachexia was diagnosed based on weight-loss or low muscularity alone. Whereas fibre type is not targeted selectively, muscle fibre size, biochemical

composition and pathway phenotype can vary according to whether the criteria for cachexia include both a measure of low muscularity and weight loss. Such findings suggest that current diagnostic criteria identify groups of patients with different skeletal muscle phenotypes. Identification of a more homogeneous patient cohort for musculo-centric intervention trials may require use of combined criteria. The findings in this Chapter where therefore used in the accurate phenotyping of patients suffering from cancer cachexia to provide novel insights into genetic targets potentially associated with the condition.

CHAPTER 5

Genetic basis of inter individual susceptibility to cancer cachexia: selection of potential candidate gene polymorphisms for association studies.

5.1 Summary

5.1.1 Aims

Part of the variation in who will and who will not develop cancer cachexia may be genetically determined. As new definitions, classifications and biological targets continue to evolve a need for the reappraisal of the literature for future candidate association studies is needed. This Chapter summarises genes identified or implicated as well as putative candidate genes contributing to cachexia, identified through diverse technology platforms and model systems and forms the basis for selection of SNPs used in Chapter 6.

5.1.2 Methods

A systematic search covering 1986–2012 was performed for potential candidate genes/genetic polymorphisms relating to cancer cachexia. All candidate genes were reviewed for functional polymorphisms or clinically significant polymorphisms associated with cachexia using the OMIM and GeneRIF databases. Pathway analysis software was used to reveal possible network associations between genes. Functionality of SNPs/genes was explored based on published literature, algorithms for detecting putative deleterious SNPs and interrogating the database for expression Quantitative Trait Loci (eQTLs).

5.1.3 Results

A total of 154 genes associated with cancer cachexia were identified and explored for functional polymorphisms. Of these 154 genes, 119 had a combined total of 281 polymorphisms with functional and/or clinical significance in terms of cachexia associated with them. Of these, 80 polymorphisms (in 51 genes) were replicated in more than one study with 24 polymorphisms found to influence two or more hallmarks of cachexia (i.e. inflammation, loss of fat mass and/or lean mass and reduced survival).

5.1.4 Conclusions

Selection of candidate genes and polymorphisms is a key element of multigene study design. The present Chapter provides a contemporary basis to select genes and/or polymorphisms for further association studies in cancer cachexia (see Chapter 6).

5.2 Introduction

The degree of cancer cachexia patients suffer is variable depending upon the phenotype and genotype of both patient and tumour. It is likely there maybe cachexia prone genotypes as well as cachexia resistant genotypes. A recent longitudinal study of patients with a variety of cancers has demonstrated some will remain stable, lose or gain skeletal muscle or adipose tissue (419), further strengthening the concept of a genetic predisposition to wasting in the presence of cancer.

Single nucleotide polymorphisms (SNPs) are the most common type of heritable and evolutionarily stable genetic variations in the population (420); other genetic polymorphisms include copy number aberrations, insertion, deletions and tandem repeats. SNPs may exert differing effects on genes leading to an aberrant gene product. Polymorphisms in promoter regions potentially contribute to differential gene expression, presumably affecting the binding of transcription factors to DNA. SNPs present in the 5' untranslated region (UTR) may alter mRNA translation, whereas SNPs in the 3' UTR could alter mRNA via post-transcriptional mechanisms such as splicing, maturation, stability and export. Polymorphisms in intronic regions may result in *cis*- or *trans* regulation of genes, unmask cryptic splice sites or promoters leading to alternative transcripts. Synonymous and non-synonymous SNPs in exons could alter protein function or activity and may introduce codon bias contributing to the relative abundance of the proteins, respectively, finally non sense mutations cause a stop altogether in the translation of mRNA (421). The genomic distribution of SNPs is not homogenous, SNPs usually occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixating the allele of the SNP that constitutes the most favourable genetic adaptation (422). Approximately 10% of all SNPs are functional, potentially altering a biological process.

The case to support a genetic predisposition to cachexia is strengthened from the known genetic contribution to the activity of a variety of key mechanisms that underlie the cachexia syndrome. In a previous systematic review on the identification of possible genetic polymorphisms involved in cancer cachexia, a total of 184 polymorphisms with functional or clinical relevance to cancer cachexia were identified in 92 candidate genes (193). Following this approach here in, 23 significant SNPs associated with cachexia based on definitions of weight loss and systemic inflammation (measured with CRP) were identified and a SNP in the SELP gene encoding for P – selectin was validated in an independent cohort. P – selectin binds to leukocytes and in certain inflammatory conditions, the plasma concentration of soluble P – selectin is highly elevated (194). By incorporating a definition based on systemic inflammation a SNP involved in the innate immune response was identified. Most of the work on identification of SNPs involved in cancer cachexia to date has led to the discovery of other SNPs involved in the innate immune system, mainly in the interleukin family of cytokines (195-202) (Table 5.1). SNPs in other biological processes have also been studied for their association with cancer cachexia (203, 204) (205) (Table 5.1). By altering the phenotype for a degree of skeletal muscle quantification, it may be possible to identify SNPs associated with muscle tissue biology.

The main aim of this thesis is to investigate further the potential genetic basis for cancer cachexia. To enable further candidate gene selection studies with larger cohorts, new targets need to be identified in order to maximise the potential for associations. An increase in sample size from before will increase the power of further studies to identify novel SNPs. To accommodate the evolving phenotype definitions and the current state of the understanding of cachexia, the search was conducted for candidate genes or pathways related to the biology of muscle, inflammation, adipose tissue, obesity, diabetes and molecular mechanisms of cancer in general, as well as factors affecting survival and prediction of outcomes following treatments. There is an express need to re-strategise the selection of candidate genes to drive association studies since “omics” approaches uncovered the hitherto unexplored biological pathways in the past five years in the realm of cancer and genetic predisposition markers for a host of diseases and traits. In view of the increased understanding of the biology of cancer cachexia, the search terms used have been amended as both the biology and classification of cancer cachexia continues to evolve. This study suggests that insights into the functional significance of the candidate gene SNPs will

help explore the putative causality of the SNPs. These approaches would likely strengthen the premise for hypothesis driven phenotype-genotype association studies for polygenic diseases/traits, vis-à-vis potential to identify variants with higher effect size and hence heritable component of the cachexia risk in individuals. Functionality of SNPs/genes was explored based on published literature, algorithms for detecting putative deleterious SNPs and databases for expression Quantitative Trait Loci (eQTLs) (423-427). Inter-individual variations in susceptibility to cancer induced cachexia and the heritable component of the genome when fully delineated would help identify for possible interventions those at risk well before the onset of clinical symptoms.

Author	SNP	Gene	Patients included	Phenotype
Solheim 2012	No associations	N/A	1853 patients with cancer at different sites, stages and with different performance status	EORTC QLQ-C30 questionnaire, question 13: “have you lacked appetite”.
Punzi 2012	rs1544410 (BsmI) rs731236 (TaqI)	VDR	43 patients with cancer from various sites and stages	Guidelines for diagnosis of cancer associated cachexia provided by the Italian Association of Medical Oncology
Tan 2012	rs6136	SELP	775 patients with UGI and pancreatic cancers	6 Phenotypes (1) >5% Weight loss (2) >10% Weight loss (3) >15% Weight loss (4 – 6) The above with CRP concentration of >10 mg/l $^{-1}$
Solheim 2011	No associations	N/A	1797 patients with cancer at different sites, stages and with different performance status	• BMI: <20 kgm^{-2} • Karnofsky score: <80 • CRP: <10 mg l^{-1} • Appetite loss: a response of little or greater on EORTC QLQ-C30 item ‘have you lacked appetite?’
Sun 2010	rs1800896	IL-10	223 Gastric cancer	
Bo 2010	rs2227306	IL-8 +781	Gastric cancer	
Jatoi 2010	rs1800629	TNF	471 patients with non-small cell lung cancer	>10% Weight loss
Rausch 2010	rs3024498	IL-10	1149 Caucasian lung cancer survivors	Lung Cancer Symptom Scale How much are you experiencing loss of appetite?
Deans 2009	rs1800896	IL-10 (-1082)	203 patients with UGI cancers	>10% Weight loss
Knoll 2008	rs2229616 Val103Ile	MC4R	509 patients with various cancers (including haematological malignancies) at various stages	(1) >10% Weight loss (Exclusivley cancer) (2) >10% Weight loss (Treatment influenced) (3) >5% Weight loss (4) >1% Weight loss (Cancer specific) or >5% treatment induced (5) No weight loss
Zhang 2007	rs1143634	IL-1β (+3954)	214 Patients with locally advanced gastric cancer	>10% Weight loss
Jatoi 2007	rs1143634	IL-1β (+3954)	44 Patients with metastatic UGI cancer	Phenotype is greater improvements in weight registered every 3 week during chemotherapy

Table 5.1 - Published associations of genetic variants in cancer cachexia

5.3 Methods

The U.S. National Library of Medicine database, MEDLINE; the Excerpta Medica database, EMBASE; Cochrane Central Register of Controlled Trials, CENTRAL; and the database of the Cumulative Index to Nursing and Allied Health Literature, CINAHL, were searched through the National Library of Health website, the Cochrane library, PUBMED (free citation database of MEDLINE), and Grey literature online.

The first aim was to identify articles with new potential candidate genes involved in the development of cancer cachexia using the key word search 'genes/genetics and inflammation OR cancer OR cachexia OR weight loss OR body composition OR survival OR muscle OR adipose to provide an update on the existing database. To further identify related genes, new candidate genes were entered into a pathway functional analysis software (Ingenuity Pathways Analysis (IPA), Ingenuity Systems, Redwood, USA).

The second and third aims were to find genetic polymorphisms with known functional or clinical significance in each of these new and existing genes using the key word search '(*gene of interest*) AND polymorphism(s). All identified candidate genes were reviewed for functional polymorphisms or clinically significant polymorphisms in terms of cachexia using OMIM, dbSNP and GeneRIF databases. The search was limited to reports within English language and a manual search of reference lists and conference proceedings followed with all cross-references screened.

5.3.1 Study selection

Published papers and or abstracts were screened after removing duplicates. Inclusion criteria were set as any study either involving genes in the selected domains or any study showing functional SNPs influencing these genes. Reasons for exclusion included studies of irrelevance to cancer cachexia, studies with no genes or functional polymorphisms identified, and complex polygenic phenotypes analysing random SNPs for association. The remaining papers were reviewed in full and only those involving genes in the development of cancer cachexia or any study showing functional SNPs influencing these genes, were included in the final analysis.

In accordance with the previous review, candidate genes were grouped based on the role their product is postulated to have in the development of cancer cachexia (193),

these being the domains of:

1. Inflammation
    a. Innate immune receptors and mediators of the immune response
    b. Cytokines
    c. Cytokine receptors and related binding proteins
    d. Acute phase protein reactants
2. Central Homeostasis
    a. Energy production
    b. Insulin like growth factors and related proteins
    c. Corticosteroid signalling proteins
3. Muscle
    a. Muscle function and structure
    b. Muscle synthesis
    c. Muscle proteolysis and degradation
4. Adipose tissue
    a. Adipogenesis
    b. Lipid turnover and transport
    c. Adipokines and adipokine receptors
5. Appetite
6. Others

The updated summary Tables of polymorphisms are presented according to each category with 'easy to see' boxes that denote whether a polymorphism has any effect on inflammation, weight/body composition (i.e. lean mass/fat mass) and cancer survival. Also added is information on ancestral allele, SNP allele, type of SNP, polymorphism reference number (rs number), and mean allele frequency (MAF) based on a population with European ancestry derived from the HapMap or dbSNP databases.

5.3.2 Pathway analysis

The methods used for pathway analysis have been described before (193). Briefly, multiple genes within a single pathway are likely to influence the development of cancer cachexia. Pathway-based analyses using the ingenuity pathway analysis (IPA) software was used to determine further related genes in single pathways. Genes containing a SNP which had been validated in at least one study were entered into

the IPA analysis tool. The IPA software measured associations of these genes with other molecules, their network interactions, and biologic functions stored in its knowledge base. These genes were entered in the IPA algorithm, which recognizes functional networks by identifying interconnected molecules, including molecules not among these genes from the IPA knowledge base. The software generates networks and calculates a score for each. This represents the approximate 'fit' between the eligible focus molecules and each network. The network score is based on the hypergeometric distribution and is reported as the –log (Fisher's exact test result).

5.3.3 Putative functions

To identify the likely effect of individual SNPs on nearby genes the Sorting Intolerant From Tolerant (SIFT; http://sift.bii.a-star.edu.sg/) program was used for non-synonymous SNPs (428), SIFT is a program that predicts whether a non-synonymous SNP leading to an amino acid substitution affects protein function or not (428). To predict whether an amino acid substitution in a protein will affect protein function, SIFT considers the position at which the change occurred and the type of amino acid change. Given a protein sequence, SIFT chooses related evolutionarily conserved proteins and amino acid residues likely critical for structure or function and obtains an alignment of these proteins with the query. Based on the amino acids appearing at each position in the alignment, SIFT calculates the probability that an amino acid at a position is tolerated conditional on the most frequent amino acid being tolerated. If this normalised value is less than a cut-off, the substitution is predicted to be deleterious (429). Many of the amino acid substitutions are well tolerated since the SNPs tend to capture subtle effects, and the ones with drastically altered functions are not to the survival advantage of the species, hence are eliminated during the course of evolution. A limitation of SIFT program is that only non-synonymous substitutions are interrogated and those in the regulatory and intronic regions (>95% of the genome) are not considered.

The SNP and copy number annotation (SCAN) database (http://www.scandb.org/newinterface/about.html) is a large-scale database providing a web-interface for easy search with a gene name or an input of SNP ID (rs#) (430). This database can use SNPs from 5' or 3' and intronic SNPs and their potential influence on the gene expression for nearby genes; gene expression if regulated on the same chromosome and within 100MB distance from the SNP could be categorized

as cis-regulatory SNPs, if not are called trans-SNPs. The SNPs are classified according to their effects on expression levels, i.e. expression quantitative trait loci (eQTLs) using SCAN database. Gene expression data and polymorphism data from HapMap population derived lymphoblastoid cell lines (Caucasian, Yoruba and Han Chinese/Japanese) are used to interrogate the cis- and trans effects from SNPs/genes. The findings for the selected SNPs for possible functional significance using this approach as well has been summarised. Generalizability of observations from genotype to expression from lymphoblastoid cell lines is not the same as correlations made with tissue specific expression, and therefore one should exercise caution in the interpretations of findings. However, several published studies attest to the utility of this approach to extrapolating genotype-phenotype correlations to be valid even to specific tissues, albeit for select candidate SNPs.

5.4 Results

A total of 281 polymorphisms in 154 genes were identified and explored for relevance to cancer cachexia (Tables, 5.2-5.16) (Figure 5.1). Of these genes, 88 were newly identified and 66 were from the previous review from our group (193). The 281 functional polymorphisms with or without clinical significance to cancer cachexia (i.e. inflammation, loss of fat mass and/or lean mass and reduced survival) were found in 119 genes with no SNPs of interest found in the remaining 35 genes. A pathway based analysis for relevance to cachexia and SNPs selected from these pathways is summarised (Table 5.3, Figures 5.2 and 5.3).

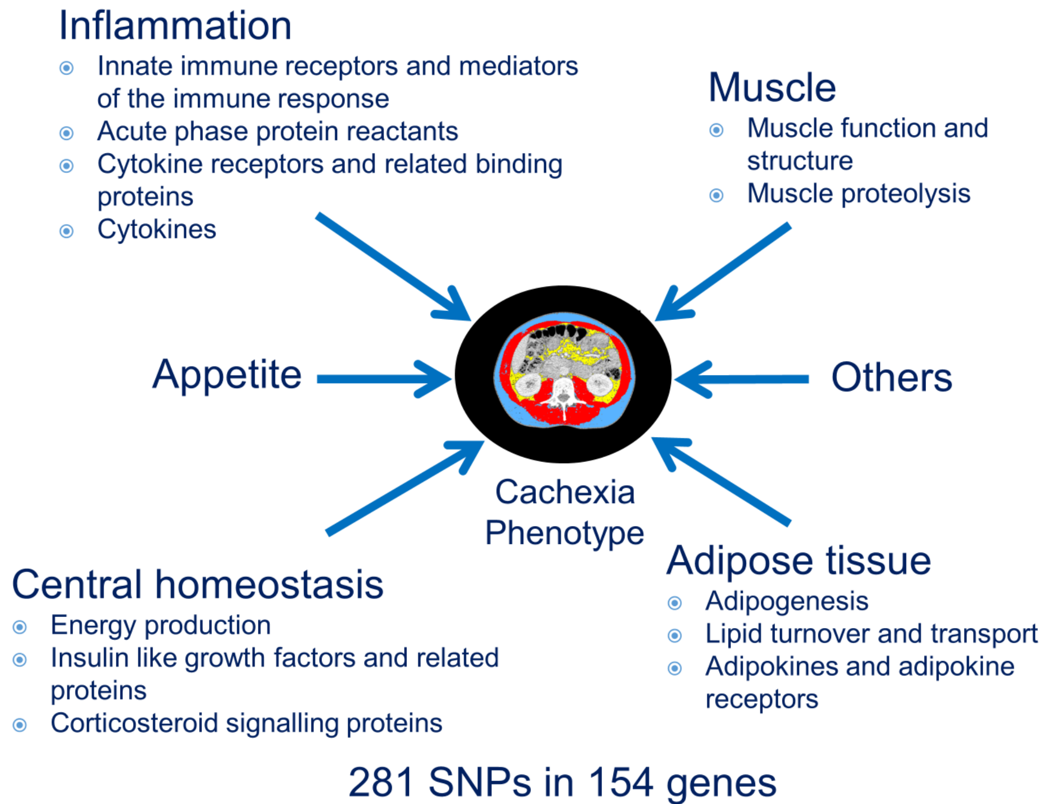


Figure 5.1 - The case to support a genetic predisposition to cachexia is strengthened from the known genetic contribution to the activity of a variety of key mechanisms that underlie the cachexia syndrome. This Chapter identified 281 single nucleotide polymorphisms in 154 candidate genes involved in these mechanisms that may contribute to the development of cancer cachexia.

5.4.1 Polymorphisms involving innate immune receptors and mediators of the immune response

(1) Inflammation

Inflammation is widely accepted to play a dominant role in the host's response to cancer and mediators regulating skeletal muscle atrophy in cachexia are thought to derive from immune or tumour cells, or the targeted tissues undergoing wasting (both adipose tissue and skeletal muscle) (431). Cancer cell rely on production of pro-inflammatory mediators for growth, protection from apoptosis, and promotion of angiogenesis/metastasis. The tumour may consequently initiate a cytokine cascade that has multiple, direct, and distant effects including the initiation of skeletal muscle protein degradation. Host immunologic response to pro and anti-inflammatory

cytokines dictates to what degree the metabolic rates are altered. In experimental models, pro-inflammatory cytokines) lead to an acute phase response and tissue catabolism (166).

(1a) Innate immune receptors and mediators of the immune response

Table 5.2 shows the genes involved in the regulation of the innate immune response and how they may generate or suppress the inflammatory response to influence the rate of cancer cachexia. Variants of these genes which may influence their function are also listed. Since the previous review, focus has spread to include new genes (AKT2, AKT3, and COX2) (286) (432) which act via signalling pathways to alter the innate immune response. AKT regulates cellular survival and metabolism by binding and regulating many downstream effectors, e.g. NF-κB and Bcl-2 family proteins (433). IGF-1 induced AKT signalling is important in both the suppression of degradation and the induction of protein synthesis (331). AKT2 has been shown to induce glucose transport, a mouse model null for AKT2 demonstrated marked growth deficiency and displayed a diabetic phenotype (434); the role of AKT3 is less clear.

Also of interest are variants coding for COX2, it is found in low levels in most cells under normal conditions but has been shown to be elevated during periods of inflammation. COX2 mediates the formation of prostaglandins from arachidonate and may have a role as a major mediator of inflammation and/or a role for prostanoid signalling (435).

New variants in the genes coding for the Toll-like receptor (TLR) family and CAMs have been found. These genes are involved in both the induction of the innate and adaptive immune responses. TLRs activate intra-cellular signals, resulting in the activation of nuclear factor NF-κB, where it up regulates the expression of other immuneregulatory substances (436), CAMs regulate migration of cells to sites of inflammation.

Table 5.2 - Summary of polymorphisms involving innate immune receptors and mediators of the immune response

Gene	SNP	Prev S/R	Gen from prev S/R	New S/R	Used in study	Validated in study	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/ Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TLR1 (193)	rs5743618 T1805G	✓	-	-	x	-	Impaired basal and lipopeptide induced NFκB signalling (437)	A	C	A = 25%	Missense Ser - Ile	↓(437)				
	rs5743611	x	-	✓	-	-	Nonsynonymous SNPs in TLR1 are associated with impaired TLR1 function, decreased cytokine responses, and predisposition to candidemia in whites.(438)	C	G	G = 4%	Missense Arg - Thr	↓(438)				✓ (437)
	rs4833095	x	-	✓	-	-	Nonsynonymous SNPs in TLR1 are associated	C	T	T = 47%	Missense Asn - Ser	↓(438)				✓ (439)

							with impaired TLR1 function, decreased cytokine responses, and predisposition to candidemia in whites.(438)									
	rs5743551	x	-	✓	-	-	Alleles -7202G and 248Ser, and the 248Ser-602Ile haplotype were associated with circulatory dysfunction among severe septic patients (0.001 ≤ p ≤ 0.022), and with reduced IL-10 (0.012 ≤ p ≤ 0.047) and elevated CRP (0.011 ≤ p ≤ 0.036) serum levels during the first week of sepsis	C	T	T = 47%	nearGene-5	↑(439)			↑ (440)	✓ (441)

							development.(439)									
TLR2	rs5743708 G2251A	✓	-	-	✓	x	Decreased NFκB activation (442)	G	A	A = 4%	Missense 753 Arg - Gln	↓				
	rs4696480 A-16934T	✓	-	-	✓	✓	Increased GT repeats in intron1 of TLR2 (443) Decreased PMBC production of TNF-α, IL-6, IL-12 (443)	T	A	A = 46%	Intron	↓				
	rs3804099	x	✓	✓	-	-	Previously explored for an association with cancer cachexia (203, 207)	C	T	C = 43%	Synonymous Ser – Ser					
	rs3804100	x	✓	✓	-	-	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 12%	Synonymous Ser – Ser					
TLR4	rs4986790 A896G	✓	-	-	✓	✓	Significantly reduced levels of NFκB translocation (444)	A	G	G = 4%	Missense 299 Asp - Gly	↓				✓

							Reduced levels of pro-inflammatory cytokines, acute phase reactants (445) Serum levels of TNF-alpha and its soluble receptors are elevated and associated with increasing BMI values in obese children. Serum cytokine levels, as modifying factors of insulin resistance, may be affected by TLR4 polymorphisms in obese children.(446)									
	rs4986791	x	-	✓	-	-	Serum levels of TNF-alpha and its soluble receptors are	C	T	T = 3%	Missense Thr - Ile	↕ (447)	↑ (448)			✓ (449)

							elevated and associated with increasing BMI values in obese children. Serum cytokine levels, as modifying factors of insulin resistance, may be affected by TLR4 polymorphisms in obese children.(446)								
	rs1554973	x	-	✓	-	-	Individuals with the TT genotype at rs1554973 (TLR4) had higher cervical concentrations of interleukin-1 beta (IL-1b) compared with those with the CT or TT genotypes	C	C/G /T	A = 15%	Intron	↑				✓ (451)

							(450)									
TLR5	rs5744168 C1174T	✓	-	-	✓	✓	Decreased NFκB activation in response to flagellin (452)	G	A	A = 4%	Stop-Gain Arg - XXX 392	↓				
LY96	**rs6472812**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 1%	Intron					
CD14	rs2569190 C-159T	✓	-	-	✓	✓	Increased levels of sCD14 (453) Lower levels of IgE (453) Higher PMBC TNF-α mRNA levels after incubation with LPS/*E.coli* (454)	G	A	A = 47%	UTR – 5	↑				
MBL2	rs7096206	✓	-	-	✓	✓	Low serum MBL levels (455)	A	G	G =18 %	nearGene-5 G-289C (Y/X)	↑			↑	
	rs5030737	✓	-	-	✓	x	Low serum MBL levels (456, 457)	G	A	A = 4%	Missense Arg - Cys 52 (D allele)	↑				✓
	rs1800450	✓	-	-	✓	x	Low serum MBL levels (456, 457)	C	T	T = 12%	Missense 54	↑				✓

											Gly - Asp (B allele)					
	rs1800451	✓	-	-	x	x	Low serum MBL levels (456, 457)	C	T	T = 6%	Missense 57 Gly - Glu (C allele)	↑				✓
ICAM1	rs5498 A1548G	✓	-	-	✓	x	G/G cells expressed lower amount of ICAM-1-mRNA than A/A cells (458) Lower fibrinogen levels (459)	A	G	G =45 %	469 Missense (Lys - Glu)				↑	
	rs5491	x	-	✓	-	-	ICAM1 DNA segment variants were associated with sICAM-1 protein level including the novel finding that levels differ by the functional variant rs5491(460)	A	T	T = 7%	Missense Lys - Met	↑	↑ (461)			✓ (461)
	rs281432	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	C	G = 47%	Intron					

VCAM1	**rs3176860**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	G=43%	Intron					
SELP	rs6136 A37674C	✓	-	-	✓	✓	Decreased serum P-selectin levels (462, 463)	T	G	G=4%	715 Missense Thr – Pro	↓			↑	✓
HSPA1L	rs2227956 C2347T	✓	-	-	✓	x	Increased plasma IL-6 levels and TNF-α levels with C allele (464)	A	G	G =12%	493 Missense Thr - Met	↓				
HSPA1B	**rs6457452**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 11%	(5’ UTR)					
AKT1	rs1130214 G205T	✓	-	-	x	-	Greater AKT1 gene expression (465)	C	A	A = 24%	(5’ UTR)			↑		
	rs11555433	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	G	?	Missense					
AKT2 / PKBB**(286)**	**rs11669332**	x	-	✓	-	-	Altered (Lower) plasma lipid	C	C/G/T	T = 12%	Intron		↓			

							concentrations(466) Individuals being homozygous for the T allele of rs11669332 (an Akt2 promoter) showed lower total/HDL cholesterol ratio and the metabolic syndrome score.									
AKT3 / PKBG(286)							None	-	-	-	-					
AGER	rs2070600	✓	-	-	✓	x	Higher levels of plasma TNF-α, serum CRP with Ser/Ser genotype (467)	C	T	T = 8%	82 Missense Gly - Ser	↑				
	rs1800625 -429T>C	x	-	✓	-	-	Leads to increased RAGE (AGER) expression (468)	G	A	G =12 %	nearGene-5					
	rs1800624 -374T>A	x	-	✓	-	-	Leads to increased RAGE	*A*	T	T = 20%	nearGene-5					

							(AGER) expression (468)									
COX2	**rs20417**	x	-	✓	-	-	Alters IL-6 levels in patients with diabetes(432)	*C*	G	G =20 %	nearGene-5	↑				✓ (469)
NFKB1	**rs3774932**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	*G*	A	A = 40%	Intron					
	rs1801	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	*G*	C	C = 36%	Intron					
NFKBIA	rs696	✓	-	-	✓	✓										

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.2 Polymorphisms of cytokines, cytokine receptors and related binding proteins

(1b) Cytokines and (1c) cytokine receptors

Mediators regulating cancer cachexia are thought to derive from immune or tumour cells, or the targeted tissues undergoing wasting (both adipose tissue and skeletal muscle). All cytokines induce immune and inflammatory responses. The resulting effects on cellular function are numerous. SNPs in genes of pro-inflammatory and anti-inflammatory cytokines are presented in Table 5.3.

Cytokines bind to their appropriate receptor to initiate a downstream cascade of intracellular signalling. The signalling leads to either potentiation of the signal and to production of similar or other cytokines and their receptors or to a suppression of the signal (102). Since the last review, the gene encoding the IL-6R protein has been shown to play an important role in the mediation of IL-6 signalling, and a genetic variant in this gene has been shown to influence the CRP level (470). Genetic variants of genes encoding cytokine receptors and related binding proteins are presented in Table 5.4.

Table 5.3 - Summary of cytokine polymorphisms

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TNF	rs1800629 G-308A	✓	-	-	✓	✓	Increased TNF-α production (471) Six fold increase in transcription of TNF-α (472)	G	A	A = 10%	nearGene-5	↑	↑			✓
	rs361525 G-238A	✓	-	-	x	-	Decreased transcriptional activity (473) Decreased PMBC production of TNF-α after stimulation with T-cell mitogens (473) Decreased insulin resistance (474)	G	A	A = 5%	nearGene-5	↓	↓			✓
	rs1799724 C-857T	✓	-	-	x	-	Increased serum TNF-	C	T	T = 10%	nearGene-5	↓	↓			

							α levels (475)									
	rs1800630 C-863A	✓	-	-	x	-	Reduced total serum IgE levels (476) Reduced serum TNF-α levels (476) 31 % decrease in transcription of TNF-α (477)	C	A	A = 15%	nearGene-5	↓	↓			✓
	rs1799964 1031 T>C	-	✓	✓	✓	✓	Appendicular skeletal muscle and arm muscle mass (CC, CT\TT) (The allele C at position −1031 was significantly associated with lower arm lean mass and ASM)	C	T	C = 20%	nearGene-5			↓ (478)		
LTA(193)	rs909253 A252G	✓	-	-	✓	✓	Increased serum TNF-α levels (479, 480)	A	G	G =40 %	Intron	↑			↓	✓

IL1A	rs17561 G4845T	✓	-	-	✓	✓	Pre IL-1α with Ala more resistant to proteases in human sera compared with Pre IL-1α with Ser (481)	C	A	A = 20%	Missense 114 Ala - Ser					
	rs180058 7 C-889T	✓	-	-	✓	✓	Significantly increased transcription al activity of the IL-1α gene with TT genotype (482) Slight increase of IL-1α mRNA and protein levels (482)	G	A	A = 25%	UTR - 5	↑				
IL1B	rs114362 7 C-31T	✓	-	-	✓	✓	Increased expression of IL-1β gene with T allele (483) Increased IL-1β production from whole	G	A	G =48 %	nearGene -5	↑			↑	✓

							blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (484) Increased transcriptional activity with -31T/-511C/-1470G haplotype (484)									
	rs16944 C-511T	✓	-	-	✓	X	Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (484) Increased transcriptional activity	A	G	A = 48%	nearGene-5	↓			↓	✓

							with -31T/-511C/-1470G haplotype (484) No significant increase in IL-1β production in response to LPS in patients homozygous for T allele (485)									
	rs1143634 C3953T (3954)	✓	-	-	✓	✓	T/T genotype associated with lower plasma levels of IL1-RA (486) Increased human amniochorion IL-1β production after stimulation with LPS (487)	G	A	A = 15%	Synonymous Phe - Phe	↑	↓		↓	✓

IL4	rs2070874 C-33T	✓	-	-	✓	✓	Increase in serum IL-4 levels (488)	T	C	T = 43%	UTR – 5	↑				
	rs2243250 C-590T						Higher gene reporter assay activity (489) Different pattern of protein binding on electrophoretic mobility shift assay (489) Higher serum levels of IgE (489)	C	T	T = 49%	nearGene -5	↑				
	rs2243248	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	G	G = 10%	nearGene -5					
IL6	rs1800795 G-174C	✓	-	-	✓	x	Lower levels of IL-6 in plasma in healthy subjects (490) Higher fasting	G	C	C = 19%	nearGene -5	↓	↑	↓		✓

							plasma insulin levels with G allele (491) Lower circulating adiponectin levels with G allele (491)									
	rs2069830 32 Pro→Ser	✓	-	-	x	-	Lower levels of IL-6 (492) Higher levels of albumin (492)	C	T	T = 4%	Missense 32 Pro - Ser	↓				
	rs2069860 162 Asp→Val	✓	-	-	x	-	Lower levels of IL-6 (492) Higher levels of albumin (492)	A	T	T = 2%	Missense Asp - Val	↓				
	rs2069835	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 6%	Intron					
	rs1554606	x	✓	-	✓	✓	Previously explored for an	T	G	T = 28%	Intron					

							association with cancer cachexia (203, 207)									
	rs2069845	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	G = 27%	Intron					
IL6R	**rs4129267**	x	-	✓	-	-	Highly associated with serum CRP levels(470)	C	T	T = 38%	Intron	↑				
	rs12750774	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	A	A = 16%	Missense Arg - Gly	↑				
IL8	rs4073 A-251T	✓	-	-	✓	x	Increased IL-8 production in whole blood stimulated with LPS with A allele (493)	A	T	T = 50%	nearGene -5	↓	↓			
	rs2227306	x	-	✓	-	-	Association of interleukin-8 gene polymorphis	C	T	T = 26%	Intron					

							m with cachexia from patients with gastric cancer(196)									
IL10	rs1800872 C-592A	✓	-	-	✓	✓	Significantly reduced levels of IL-10 (494)	G	T	T = 41%	nearGene -5	↑				
	rs1800896 G-1082A	✓	-	-	✓	✓	Increased levels of IL-10 with -1082GG (495) -1082GG associated with increased CRP (496)	T	C	C = 41%	nearGene -5	↕			↑	
	rs3024498	x	-	✓	-	-	Relationship between cytokine gene single nucleotide polymorphisms and symptom burden and quality of life in lung cancer survivors (199)	T	C	C = 11%	UTR - 3					

IL12B	rs3213113 A16974C	✓	-	-	x	-	Higher IL-12 secretion levels from LPS and PPD stimulated PBMCs in C/C genotype (497)	T	G	G = 1%	UTR – 3	↑				
	rs1368439	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	G	G = 8%	UTR – 3					
IL13	rs1800925 C-1055T	✓	-	-	✓	✓	Decreased inhibition of IL-13 production upon stimulation with anti-CD2 (498) Increased binding of nuclear proteins (498)	T	C	T = 25%	nearGene-5	↓				
IL18	rs187238 G-137C	✓	-	-	✓	✓	Increased IL-18 production from LPS and	C	G	G =23 %	nearGene-5	↓				

							A23187 + PMA stimulated monocytes in 105AA and -137GG (499)									
	rs5744256 C/T	✓	-	-	✓	✓	Reduced serum concentrations of IL-18 (500)	A	G	G =10 %	Intron	↓				
	rs1946519 G-9731T	✓	-	-	✓	✓	Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (501)	A	C	C = 46%	nearGene -5	↑	↓			
	rs2043055 T-5848C	✓	-	-	✓	✓	Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (501)	A	G	G =42 %	Intron	↑	↓			
	rs549908 A105C	✓	-	-	✓	✓	Increased IL-18 production from LPS	T	G	G =23 %	Synonymous Ser - Ser	↑	↓			✓ (499

							and A23187 + PMA stimulated monocytes in 105AA and -137GG (499) Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (501)								), (501)
	rs360729 T8855A	✓	-	-	✓	✓	Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (501)	A	T	T = 24%	Intron	↑	↓		
	rs3882891 A11015C	✓	-	-	x	-	Haplotype of common alleles (GTATA) associated with significantly	G	T	G =50 %	Intron	↑	↓		

							lower IL-18 (501)									
IFNA2	**rs624704**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	G	G = 49%	nearGene -3					
	rs632941	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	G	A = 49%						
IFNG	rs2430561 T874A	✓	-	-	✓	✓	Decreased mRNA levels of IFN-γ and IL-6 (502) Lower frequency of CRP elevation (502) Preferential binding of NFκB to T allele (503)	T	A	A = 20%	Intron	↓				✓
	rs2193049	x	✓	-	✓	✓	Previously explored for an association with cancer	C	G	C = 29%						

							cachexia (203, 207)									
	rs2069727	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 27%	nearGene -3					
	rs2069709 G-179T	✓	-	-	✓	x	Increased reporter gene assay activity in response to TNF-α (504)	C	A	A =<1 %	nearGene -5	↑				
TGFB1	rs1982073 / rs1800470 T29C	✓	-	-	x	-	Significantly higher fasting insulin values as well as higher insulin-resistance in heterozygotes (505)	G	A	G =40 %	Missense Pro - Leu		↑			
	rs1800469 C-509T	✓	-	-	✓	x	-509C exclusively binds AP1 which downregulates expression of TGF-β1	G	A	A = 36%		↑			↑	

							promoter (506)									
GDF15	rs1058587	✓	-	-	✓	✓										
MIF	rs755622 G-173C	✓	-	-	✓	✓	Higher serum and synovial fluid levels of MIF (507)	C	G	C = 27%	ncRNA	↑			↕	
CCL2	rs1024611 A-2518G (A-2578 G)	✓	-	-	✓	x	Increased transcription of CCL2 gene with reporter gene assay (508) Associated with higher serum MCP-1 level (509)	A	G	G =40 %		↑				✓
CCL5	rs2107538 G-403A	✓	-	-	✓	x	G allele associated with higher serum levels of RANTES (510) Serum RANTES correlates with CRP levels (510)	T	C	T = 28%	nearGene -5	↑				

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

Table 5.4 - Summary of polymorphisms of cytokine receptors and related binding proteins

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele/s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
TNFRSF1A	rs4149570 G-329T	✓	-	-	✓	✓	T allele results in repression of TNFSFR1A expression (511)	G	A	A = 31%	nearGene-5	↓	↓			
	rs767455 A36G	✓	-	-	✓	✓	Increased TNFR1 levels with 36GG (512)	T	C	C = 33%	Synonymous Pro - Pro	↑	↑			
TNFRSF1B	rs1061622	✓	-	-	✓	✓	Significantly lower capability to induce TNFR2-mediated NFκB activation (513)	T	G	G =20%	Missense Met - Arg	↓				
	rs1061624 G1663A	✓	-	-	x	-	Increased gene transcription with 1663G, 1668T, 1690C	A	G	G =48%	UTR – 3	↓	↓			

							haplotype (514)									
	rs5030792 T1668G	✓	-	-	x	-	Increased gene transcription with 1663G, 1668T, 1690C haplotype (514)	T	G	G = 4%	UTR – 3					
	rs3397 T1690C	✓	-	-	✓	✓	Increased gene transcription with 1663G, 1668T, 1690C haplotype (514)	T	C	T = 45%	UTR – 3	↑	↑			
	rs496888	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	C = 29%	Intron					
	rs976881	x	✓	-	✓	✓	Previously explored for an association with cancer	C	T	T = 27%	Intron					

							cachexia (203, 207)									
	rs1061631	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A=13 %	UTR – 3					
IL1R1	**rs2228139**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	G	G=5 %	Missense Ala - Gly					
IL1RN	**rs4251961**	x	-	✓	-	-	The minor allele on this locus was associated with higher serum CRP and, ex vivo, with decreased leukocyte production of IL-1 receptor antagonist protein in	T	C	C = 23%	nearGene -5	↕				✓ (516)

							response to an inflammatory stimulus.(515)									
IFNGR1	rs2234711 T-56C	✓	-	-	x	-	Lower transcription of IFNGR1 promoter with reporter gene assays (517)	G	A	G =44%	UTR – 5	↓				
	rs9389484	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 24%	Intron					
	rs7749390	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	G =45%	Intron					
LITAF	**rs4280262**	x	✓	-	✓	✓	Previously explored for an	C	T	C = 12%	Missense Ile - Val					

							association with cancer cachexia (203, 207)									

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.3 Polymorphisms of acute phase proteins

(1d) Acute phase protein reactants

An organism responds to the presence of acute infection, tissue injury, trauma or surgery by mounting an acute phase response (APR), this is designed to help limit tissue injury by the increased synthesis of key defence / repair proteins by the liver. However, in certain circumstances when dietary protein intake is limited and the APR is prolonged or severe, an APR can exacerbate muscle wasting by increasing the demands for certain amino acids to support increased hepatic export protein synthesis, they have also been shown to be predictors of adverse outcomes in cancer patients (518). C-reactive protein (CRP) is an acute phase protein which influences the immune system. CRP genetic variants have been increasingly studied since the last review and some of these have been replicated in other studies. These represent promising targets for future genetic studies into links with degrees of cachexia. Variants in genes coding for APPR are shown in Table 5.5.

Table 5.5 - Summary of polymorphisms of acute phase proteins

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
CRP (193)	rs1800947 G1059C	✓	-	-	✓	✓	Lower plasma CRP concentration with C/C genotype (519, 520)	C	G	G = 4%	Synonymous Leu - Leu					✓
	rs2794521 A1009G (-717)	✓	-	-	x	-	Lower serum concentration on CRP (521)	T	C	C = 21%	nearGene -5					
	rs1130864 C1444T	✓	-	-	✓	x	Higher CRP concentrations (522, 523)	G	A	A = 21%	UTR – 5					✓
	rs3093059 -757	x	-	✓	-	-	T Allele associated with higher serum CRP concentrations(524)	A	G	G =15 %	nearGene -5	↑				
	rs3093062 -409	x	-	✓	-	-	G Allele associated with higher serum CRP concentrations(524)	C	T	T = 4%	nearGene -5	↑				
	rs3091244 -390 1440	x	-	✓	-	-	T Allele associated with higher serum CRP	G	A	A = 25%	nearGene -5	↕				✓ (526, 527)

							concentrations(524, 525)									
	rs1417938 +29 1919	x	-	✓	-	-	A Allele associated with higher serum CRP concentrations(524)	T	A	A = 20%	Intron	↕				✓ (526),[93]
	rs3093066 +219 3006	x	-	✓	-	-	C Allele associated with higher serum CRP concentrations(524)	G	T	T = 7%	UTR – 3	↑				✓ (526)
	rs1205 +1846 3872	x	-	✓	-	-	C Allele associated with higher serum CRP concentrations(524)	C	T	T = 37%	UTR – 3	↑				✓ (526)
	rs3093068 +2911	x	-	✓	-	-	G Allele associated with higher serum CRP concentrations(524)	G	C	C =13 %		↑				
	rs2794520	x	✓	✓	✓	✓	Highly associated with serum CRP levels(528)	C	T	T = 37%		↑				
	rs2808629	x	-	✓	-	-	Highly associated with serum CRP levels(528)	A	G	A = 37%		↑				

	rs3122012	x	-	✓	-	-	Highly associated with serum CRP levels(470)	T	C	C = 20%		↑				
	rs4131568	x	-	✓	-	-	Highly associated with serum CRP levels(470)	C	T	T = 20%		↑				
	rs3116654	x	-	✓	-	-	Highly associated with serum CRP levels(470)	A	G	G = 7%		↑				
	rs863013	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	T	T = 24%		↑				
ALB	**rs3775485**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	T	A = 50%	Intron					
	rs962004	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	T/G	50%	Synonymous Leu - Leu					
FGB(193)	rs1800790 G-455A	✓	-	-	x	-	Increased plasma fibrinogen levels (529)	G	A	A = 15%	nearGene-5		↑			

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.4 Polymorphisms regulating energy production

(2) Central Homeostasis

(2a) Energy production

The human body responds to stress with resilience and ultimately aims to maintain homeostasis. The mechanisms to respond to injury can ultimately prove detrimental to the host. For example, although the hepatic acute phase protein response is useful in acute injury (e.g., haemostasis and wound healing), if the response is prolonged and potentially futile (as in advanced cancer), then what results is an accelerated loss of skeletal muscle and excess morbidity and mortality. Depending on which type of cancer, patients with cachexia have been observed to have much higher resting energy expenditure (REE) (530). Gene polymorphisms in the regulatory pathways controlling energy intake and expenditure are discussed below. This domain also explores genes involved in growth and development, and metabolic pathways common to both muscle and adipose tissues.

Table 5.6 shows the genes involved in energy production, these consist mainly of the uncoupling proteins. Uncoupling proteins are transporters present in the mitochondrial inner membrane that mediate a regulated discharge of the protein gradient that is generated by the respiratory chain. This serves to regulate functions such as thermogenesis, maintenance of the redox balance, or reduction in the production of reactive oxygen species (531). No new functional SNPs were found in this group during this review.

Table 5.6 - Summary of polymorphisms regulating energy production

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
UCP 1	rs10011540 A-112C	✓	-	-	x	-	Increased insulin resistance (532)	T	G	G =10 %	nearGene -5					
	rs1800592 A-3826G	✓	-	-	✓	✓	Reduced mRNA expression of UCP1 (533)	T	C	C = 42%			↑			
	rs12502572	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	A = 47%	Intron					
UCP 2	rs659366 G-866A	✓	-	-	✓	✓	Increased UCP2 mRNA expression and transcriptional activity (534, 535) Increased insulin resistance with A/A	C	T	T = 42%	nearGene -5		↓			✓

							genotype (536)									
	rs660339 C164T	✓	-	-	✓	✓	Increased plasma leptin (537) Increased fasting insulin with Val/Val genotype (538)	G	A	A = 43%	Missense Ala - Val		↑			
UCP 3	rs1800849 C-55T	✓	-	-	✓	✓	Higher skeletal muscle UCP3 mRNA expression (539)	G	A	A = 20%	UTR – 5		↓			✓
TPI1	rs28934569 G/C (104 Glu→ Asp)	✓	-	-	x	-	Associated with TPI deficiency (540, 541)	G						↓		✓

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.5 Polymorphisms of insulin like growth factors and related proteins

(2b) Insulin like growth factors and related proteins

The IGF signalling pathway consists of two main ligands (IGF-1 and IGF-2), two cell surface receptors (IGFR1 and IGFR2), and six high – affinity IGF-binding proteins (IGFBP1-6) (542). The predominant regulator of skeletal muscle hypertrophy is through stimulation of the PI3K/AKT pathway by insulin or IGF-1 (329-331). Mice in which AKT is transgenically expressed and inducibly activated in skeletal muscle demonstrate dramatic hypertrophy upon the activation signal (332-334), helping to prove that AKT is the pathway that is sufficient to mediate hypertrophy downstream of IGF1 upregulation. Activation of AKT leads to an increase in the mTOR/p70S6K pathways and a rise in protein synthesis. As well as inducing protein synthesis, IGF1 can inhibit skeletal muscle atrophy. In the presence of up regulated IGF1 signalling, the atrophy genes MuRF-1 and MAFbx/atrogin-1 are actively inhibited (83). A number of new genetic variants for IGF-1 and IGF-1R have been discovered recently and are included in Table 5.7.

Table 5.7 - Summary of polymorphisms of insulin like growth factors and related proteins

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
IGF1(193)	**rs6220**	x	-	✓	-	-	Common genetic variation in the IGF-1 gene is related to circulating levels of IGF-1.(543)	G	A	G =35 %	UTR – 3	↑				✓(544)
	rs7136446	x	-	✓	-	-	Genotype CC of rs7136446 associated with higher body fat and increased maximal force production(545) Significantly associated with elevated levels of IGF-I(543)	T	C	C = 29%	Intron	↑	↓	↑		✓ (543), (545),
	rs972936	x	-	✓	-	-	The rs972936	T	C	T = 23%	Intron					

							GG genotype was associated with enhanced circulating levels of IGF-I.(546)									
	rs35767	x	-	✓	-	-	SNP (rs35767) showed borderline evidence of higher levels of IGF1 in carriers(547)	A	G	A = 28%	nearGene -5	↑				
	rs11111 272	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	C	G =36 %	Intron					
	rs10735 380	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	A	G= 21%	Intron					
IGF2	rs680	✓	-	-	✓	✓	Higher serum IGF-	T		T = 28%	UTR – 3		↓			

	G820A (G17200 A)						II concentration in A/A genotype (548)									
IGFBP3	rs2854746 C2133G	✓	-	-	x	-	Increased IGFBP3 levels (549, 550)	G	C	G =49 %	Missense Ala – Gly				↓	✓
	rs2854744 C-202A	✓	-	-	x	-	Increased IGFBP3 levels (550, 551)	G	T	G =46 %	nearGene -5				↓	✓
	rs2453839	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 23%	Intron					
	rs3110697	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	G	A = 40%	Intron					
IRS1	rs1801278 971 Gly→Arg	✓	-	-	x	-	Decrease in IRS-1 associated PI3-kinase activity (552) Decrease in binding of	C	T/G/A	T = 5%	Missense Gly – Arg		↓			

							the p85 regulatory subunit of PI3-kinase to IRS-1 (552) Decrease in incorporation of thymidine into DNA (552)									
	rs1025333	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	T	A =17 %	Intron					
	rs2234931	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 5%	Synonymous Gly - Gly					
DIO1	rs11206244 C785T	✓	-	-	✓	✓	Lower activity of D1, higher levels of free IGF-I with haplotype 785T,	C	T	T = 29%	UTR – 3			↑		

							1814A (553)								
	rs11206 246	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	T = 9%					
IGF1R(2 86)	**rs61740 868**	x	-	✓	-	-	Damaging alteration causing derangeme nt in receptor function (554)	C	T	U/K	Missense Arg – Cys				
	rs45578 132	x	-	✓	-	-	Damaging alteration causing derangeme nt in receptor function (554)	T	C	U/K	Missense Val – Ala				
	rs45553 041	x	-	✓	-	-	Damaging alteration causing derangeme nt in receptor function (554)	G	A	U/K	Missense Arg – His				
	rs45504 297	x	-	✓	-	-	Damaging alteration	T	C	U/K	Missense Leu - Pro				

							causing derangement in receptor function (554)									
	rs45524940	x	-	✓	-	-	Damaging alteration causing derangement in receptor function (554)	A	G	U/K	Missense Thr - Ala					
	rs45512296	x	-	✓	-	-	Damaging alteration causing derangement in receptor function (554)	G	A	U/K	Missense Arg – His					
	rs45437300	x	-	✓	-	-	A nonsense SNP implicated in receptor alteration (554)	T	A	U/K	Stop-Gain Leu - XXX					
	rs2229765	x	-	✓	-	-	Affecting splicing region associated with several diseases. (554)	A	G	G =37 %	Cds – synon Glu - Glu	↑				✓ (554), (555)

	rs55895 813	x	-	✓	-	-	In the intronic region of IGF1R, predicted to affect splicing regulation (554)	A	G	U/K	Intron affecting splicing				
	rs36108 138	x	-	✓	-	-	In the intronic region of IGF1R, predicted to affect splicing regulation (554)	C	A	A =0.7 %	Intron affecting splicing				
	rs45495 500	x	-	✓	-	-	In the intronic region of IGF1R, predicted to affect splicing regulation (554)	C	T	U/K	Intron affecting splicing				
SGK1(2 03, 207)	**rs17439 66**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	G	G =24 %	Intron				✓ (203, 207)

	rs9402571	x	-	✓	-	-	The rs9402571 SGK genotype associates with increased insulin secretion in lean non-diabetics (556)	T	G	G =26 %						

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.6 Polymorphisms of corticosteroid signalling proteins

(2c) Corticosteroid signalling proteins

Corticosteroids are steroid hormones secreted by the adrenal cortex and lead to changes in a number of organ systems. They exert their main effects on stress response, immune response and regulation of inflammation, carbohydrate metabolism and protein catabolism. A number of new genes have been included, the corticotropin-releasing hormone receptor 1 (CRHR1) binds to corticotropin-releasing hormone, a potent mediator of endocrine, autonomic, behavioural, and immune responses to stress. A number of polymorphisms in this gene have been shown to effect circulating levels of CRP and ICAM-1 (557). The glucokinase regulatory protein (GCKR) binds and moves glucokinase (GK), thereby controlling both activity and intracellular location (558) of this key enzyme of glucose metabolism (559). Genetic variants of this protein have also been linked with alterations in circulating CRP levels (470). The genetic variants of the components in the mechanism of corticosteroid signalling are examined in Table 5.8.

Table 5.8 - Summary of polymorphisms of corticosteroid signalling proteins

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
GCCR	rs6195 A1220G	✓	-	-	x	-	Associated with increased glucocorticoid sensitivity, resulting in significantly increased transactivating capacity (560)	U/K	U/K	U/K	Missense 363 Asn→Ser		↑			✓
	rs6189 G/A	✓	-	-	x	-	Lower CRP levels (561) Associated with a relative resistance to glucocorticoids (562)	C	T	T = 1%	Missense22 Glu→Glu	↓	↓	↑		
	rs6190 G/A Collectively known as ER22/23K polymorphism	✓	-	-	x	-	Lower CRP levels (561) Associated with a relative	C	T	T = 1%	Missense 23 Arg→Lys					

							resistance to glucocorticoids (562)									
HSD11B1	rs12086634 T83597G	✓	-	-	✓	✓	Lower transcriptional activity with reporter gene assays (563) Lower early morning cortisol levels and higher cortisol response to ACTH (564)	T	G	G=0.1 %	Intron 3		↓			
	rs2236903	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	A	T = 45%	Intron					
NR3C1(203, 207)	**rs6195**	x	-	✓	-	-	Reduced first-phase glucose-stimulated insulin secretion	A	G	G = 5%	Missense Asn – Ser	↓	↓			✓ (565), (566)

							and disposition index in women, but not in men.(565) Associated with enhanced glucocorticoid sensitivity (560).									
	rs6198	x	-	✓	-	-	The G allele of the 9beta polymorphism was associated with lower serum cortisol levels in women (567)	T	C	C = 9%	UTR-3		↓			
	rs41423247	x	-	✓	-	-	Female BclI GG homozygotes had higher serum cortisol levels(567)	G	C	C = 28%	Intron		↑			
	rs11749561	x	✓	-	✓	x	Previously explored	T	C	C = 37%	Intron					

							for an association with cancer cachexia (203, 207)									
CRHR1	**rs16940665**	x	-	✓	-	-	Associated with approximately 4% lower levels of ICAM-1(557)	C	T	C = 12%	Cds-synon (Thr-Thr)	↓				
	rs17689824	x	-	✓	-	-	Associated with approximately 4% lower levels of ICAM-1(557)	T	C	T = 12%	Intron	↓				
	rs7209436	x	-	✓	-	-	Associated with approximately 16% lower levels of CRP(557)	C	T	C = 47%	Intron	↓				✓ (557), (568)
	rs110402	x	-	✓	-	-	Associated with approximately 16% lower levels of CRP(557)	G	A	G =46%	Intron	↓				

GCKR	**rs780094**	x	-	✓	-	-	Highly associated with serum CRP levels(470)	C	T	T = 39%	Intron	↑	↑			✓ (470), (569)
	rs1260333	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	A	A = 45%		↑				✓ (470), (570)
	rs780106	x	-	✓	-	-	Highly associated with serum CRP levels(470)	C	A	C = 40%	Intron	↑				
	rs1647266	x	-	✓	-	-	Highly associated with serum CRP levels(470)	C	T/A/G	C = 40%	Intron	↑				
	rs13013484	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	A	G =46%	U/K	↑				
HNF1A	**rs7310409**	x	-	✓	-	-	Highly associated with serum CRP levels(470)	A	TCG	A = 39%	Intron	↑				✓ (470), (570)
	rs1169300	x	-	✓	-	-	Highly associated	A	G	A = 33%	Intron	↑				

							with serum CRP levels(470)									
	rs2464196	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	A	A = 33%	Missense (Ser-Asn)	↑				
	rs1169302	x	-	✓	-	-	Highly associated with serum CRP levels(470)	T	ACG	G =48%	Intron	↑				

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.7 Polymorphisms regulating muscle biology

(3) Muscle

In healthy adults, skeletal muscle mass is maintained within relatively narrow limits, reflecting a dynamic balance between protein synthesis and degradation. A predominance of either will result in muscle hypertrophy or atrophy. Even small changes in protein synthesis or degradation will lead to large protein deficits over time due to the continuous process of protein turnover. In cancer cachexia, there is ongoing debate as to whether a reduction in protein synthesis, an increase in protein degradation or a combination of both is more relevant. Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients' function and quality of life and is clearly associated with a poor outcome (11, 12, 69). Highlighted below are the genetic variations that affect the structure and function of muscle as well as those that regulate muscle synthesis and degradation.

(3a) Muscle structure and function

IL-15 signals through IL-15 receptor alpha (IL-15RA) and is found in abundance in skeletal muscle. IL-15 is shown to be anabolic, marked by an increase in myosin heavy chain accumulation (272). ACTN3 (Alpha-actinin 3) binds to actin at the Z-line within muscle fibres and acts to anchor actin filaments. Polymorphisms in ACTN3, IL15 and IL15RA are shown in Table 5.9.

Much recent work has focused on polymorphisms associated with alterations fat free mass in the gene encoding the vitamin D receptor (VDR). Promising new variants encoding for this protein are also included in supplementary Table 8. Inhibin β C (INHBC) is a newly added gene, it is part of the transforming growth factor β pathway regulating myostatin (a negative regulator of muscle mass). Polymorphisms identified with this gene are shown in Table 5.9. Another significant new addition to the genes implicated in cancer cachexia is thyrotropin-releasing hormone receptor (TRHR). Thyrotropin-releasing hormone is released by the anterior pituitary and acts on a number of tissues, including muscle to influence metabolic rates. Genetic variants are listed in Table 5.9.

New variants in the ACE gene have also been included. Alterations in this gene have led to differences in oxygen carrying capacity of muscles as well as increased vasoconstriction in blood vessels within the muscle architecture(571). Acute and chronic exposure to angiotensin II in animal models are associated with weight loss and enhanced protein breakdown in skeletal muscle (572). Genetic variants are listed in Table 5.9.

Table 5.9 - Summary of polymorphisms regulating muscle structure and function

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
ACTN3	rs1815739 C/T (577 Arg→TER) R577X	✓	-	-	✓	✓	Influences the response of quadriceps muscle power to strength training in older adults.(573) Polymorphism may influence declines in certain measures of physical performance with aging in older white adults(574)	C	T	T = 37%	U/K			↓		
VDR	rs2228570 T/C	✓	-	-	x	-	Greater transcriptional activation of reporter genes *in vitro* (575)	A	T/C/G	A = 34%	Missense 1 Met→Thr			↓		
	rs2239179	x	-	✓	-	-	Associated with approximately 7% higher	T	C	C = 34%	Intron	↑				

							levels of fibrinogen									
	rs2228570	x	-	✓	-	-	*FokI* in exon 9. Quadriceps isometric and concentric strength were higher in female f/f (T) homozygotes compared to F(C) allele carriers(576) The FokI VDR polymorphism is associated with skeletal muscle strength in both COPD patients and control subjects, whereas the BsmI polymorphism is associated with strength only in patients(577) FokI polymorphism was significantly associated	A	T/C/G	A = 34%	Missense T-C (Met-Thr) T-A (Met-Lys) T-G (Met-Arg)			↓		✓ (576), (577), (578)

							with FFM and sarcopenia in older Caucasian men(578)										
	rs1544410	✓	-	-	✓	x	*BsmI* in intron 8. Male Bt/Bt (A/C – A/C) homozygotes had higher isometric quadriceps strength at 150 degrees and higher concentric quadriceps strength than bT (G/T) allele carriers(576) The VDR BsmI polymorphism is associated with muscular strength in elderly men(579) There was an association between th VDR BsmI variation and muscle strength in	C	T	T = 25%	U/K			↑		✓ (576), (579), (580)

							elderly non-obese women(580)									
	rs731236	x	-	✓	-	-	*TaqI* synonymous SNP in exon 9. Male Bt/Bt (A/C – A/C) homozygotes had higher isometric quadriceps strength at 150 degrees and higher concentric quadriceps strength than bT (G/T) allele carriers(576)	A	G	G =27 %	Cds-synon (Ile-Ile)			↑		
ACE**(581)**	**rs4291 A-240T**	x	-	✓	-	-	The T-allele of this SNP was associated with depression and depressed T-allele carriers showed higher ACE serum activity and HPA-axis hyperactivity(582)	A	T	T = 35%	Near-gene 5					

	rs4343 A2350G	x	-	✓	-	-	Associated with altering circulating levels of ACE(583)	A	G	G =38 %	Cds-synon (Thr-Thr)					✓ (583), (584)
	rs4295	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	G	C = 36%	Intron					
	rs4329	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 48%	Intron					
	rs4341	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	G	G =48 %	Intron					
	rs4362	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 44%	Cds-synon (Phe-Phe)					
CNTF(585)	**rs1800169**	x	-	✓	-	-	G/A genotype possess significantly greater muscular strength and	G	A	A = 12%	Intron		↑	↑		✓ (586), (587)

							muscle quality at relatively fast contraction speeds than do G/G individual(586)									
INHBC(588)	**rs2854464**	x	-	✓	-	-	rs2854464 AA individuals were ~2% stronger than G-allele carriers(588)	G	A	G =36 %	UTR-3					
TRHR(589)	**rs16892496**	x	-	✓	-	-	Subjects carrying unfavorable genotypes at rs16892496 and rs7832552 had, on average, 2.70 and 2.55 kg lower LBM, respectively, compared to those with alternative genotypes(589)	A	C	C = 35%	UTR-3			↓		✓ (589), (590)
	rs7832552	x	-	✓	-	-	Subjects carrying unfavorable genotypes at	C	T	T = 34%	Intron			↓		

							rs16892496 and rs7832552 had, on average, 2.70 and 2.55 kg lower LBM, respectively, compared to those with alternative genotypes(589)									
CNTFR(585)	**C-1703T rs3808871**	x	-	✓	-	-	T-allele carriers of the C-1703T polymorphism in CNTFR exhibited higher strength levels for multiple measures compared to C/C homozygotes, including all knee flexor torque values(591)	G	A	A = 19%	U/K			↑		✓ (591), (592)
	T1069A rs2070802	x	-	✓	-	-	A-allele carriers at the T1069A locus in CNTFR	T	A	T = 9%	Intron			↓		

							exhibited lower concentric knee flexor isokinetic and isometric torque compared to T/T homozygotes(591)									
CRHR2(593)							None									
MKNK2 / MNK2(594)							None									
HINT3(177)							None									
TIE1(89)							None									

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

(3b) Muscle synthesis

The main signalling pathway for muscle synthesis is via the IGF-1/PI3K/AKT axis. Polymorphisms relating to these can be found in the *insulin like growth factors and related proteins* section (Table 5.7). mTOR, RUNX1, Phosphoinositide 3-kinase (PIK3) and various isoforms have been implicated in hypertrophy signals of muscle mass (286) and have been added to the list of genes, however very few studies have looked into how variants effect structure and function of these gene products. A number of genes from expression arrays were also included as *cis*- or *trans* acting polymorphisms on the genome may be affecting their expression. Genetic variants influencing function can be found in Table 5.10

Table 5.10 - Summary of polymorphisms regulating muscle synthesis

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/ Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
PIK3CA(286)	**rs17849079**	x	-	✓	-	-	May play a role in increased PI3K signalling(595)	C	T	2%	Cds – synon Thr - Thr			↑		
PIK3CB(286)	**rs361072**	x	-	✓	-	-	Minor G allele of PIK3CB rs361072 associates with decreased muscle p85alpha:p110 beta ratio and lower hepatic glucose production at high plasma insulin levels(596) C allele of rs361072 is a causal variant capable of attenuating insulin resistance in obese children through increased	A	G	G =30 %	nearGene-5		↑			✓ (596), (597)

							expression of p110beta.(597)									
mTOR(286)	**rs118203426**	x	-	✓	-	-	Increased activation of mTOR signalling(598)	A	C	U/K	Missense Met – Arg			↑		
	rs2536	x	-	✓	-	-	Altered lymphocyte function(599)	T	C	C = 7%	UTR-3	↓				
HSP90AA1(600) / WDR20	**rs1190584**	x	-	✓	-	-	Causes a change in the phosphorylated:total AKT1 ratio(600)	C	T	C = 18 %	Intron					
RUNX1(286)	**rs2834650**	x	-	✓	-	-	Increased activity following hypoxic trigger(601)	C	T	T = 12 %	Intron					
	rs12626613	x	-	✓	-	-	Associated with changing circulating IgE levels(602)	C	G	G =18 %	nearGene-5	↓				
	rs2071029	x	-	✓	-	-	Associated with changing circulating IgE levels(602)	C	T	T = 18 %	nearGene-5	↓				
PHLPP2	**Ser-1016**	x	-	✓	-	-	Ser-1016, has impaired phosphatase activity toward the substrate Akt(603)									

SKI(286)							None									
Pax3							None									
Pax7							None									
Myogenin							None									
MyoD							None									
Myf5							None									
Myf6							None									
FOXK1(286)							None									
NTF3(604)							None									
SOD-1(604)							None									

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

(3c) Muscle proteolysis

In atrophying muscles, the ubiquitin ligases, MuRF1/atrogin-1 and MAFbx, are induced and this response is necessary for rapid atrophy. FOXO isoforms 1 and 3 are known to act on MuRF1 promoter to cause MuRF1 transcription and this leads to dramatic atrophy of myotubes and muscle fibres (286, 605). IKKα also influences the Ubiquitin Proteasome Pathway (UPP) and a number of functional polymorphisms are listed in supplementary Table 10. Another gene encoding for tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) is an important adaptor protein involved in receptor-mediated activation of various signaling pathways in response to cytokines and bacterial products. TRAF6 also possesses E3 ubiquitin ligase activity causing lysine-63-linked polyubiquitination of target proteins (606).

A more recent factor, which has captured the attention of many investigators, is the TGF-β family member, myostatin. Genetic null animals for myostatin, demonstrate dramatic muscle hypertrophy (288). Myostatin is synthesised and secreted mainly from skeletal muscle cells. Myostatin acts firstly by signalling through the activin type II receptor (ACVR2B), which then recruits an Alk family kinase, resulting in the activation of a SMAD2 and SMAD3 transcription factor complex (289, 290).

STAT3 has recently been shown to influence muscle wasting by altering the profile of genes expressed and translated in muscle such that amino acids liberated by increased proteolysis in cachexia are synthesised into acute phase proteins and exported into the blood (286).

Peroxisome proliferator-activated receptor gamma (PPARγ) and its response gene, Acyl CoA synthetase 5 (ACSL5), have an important role in fatty acid metabolism and may affect weight loss in response to caloric restriction. Genetic polymorphisms have demonstrated reduced ACSL5 mRNA in skeletal muscle biopsies (607). Polymorphisms in all these genes are presented in Table 5.11.

Table 5.11 - Summary of polymorphisms regulating muscle proteolysis

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/ Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
STAT3(286)	**rs113994135**	x	-	✓	-	-	Cells have defective responses to cytokines, including interleukin (IL)-6 and IL-10, and the DNA-binding ability of STAT3 in these cells was greatly diminished.(608, 609) Renner et al. (2007)(610) noted that arg382, which is highly conserved and directly involved in DNA binding, accounted for nearly half of the **STAT3** mutations identified by Minegishi et al. (2007) and Holl	G	A	U/K	Missense ARG382 TRP (1144C-T **)**	↑				✓ 151 152

							and et al. (2007).									
	rs113994136	x	-	✓	-	-	As above(608, 609)	C	A/T	U/K	Missense ARG382GLN (1145G-A)	↑				✓ [151] [152]
	rs113994137	x	-	✓	-	-	As above(608, 609)	C	T	U/K	Missense ARG423GLN (1268G-A)	↑				✓ [151] [152]
	rs113994139	x	-	✓	-	-	As above(608, 609)	C	T	U/K	Missense VAL637MET (1909G-A)	↑				✓ [151] [152]
	rs744166	x	-	✓	-	-	Minor G allele carriers for for this SNP had increased risk of abdominal obesity compared with noncarriers (611)	A	G	G =45 %	Intron C.-23-13644T>C	↑	↑			
	rs8069645	x	-	✓	-	-	minor G allele carriers for this SNP had increased risk of abdominal obesity	A	G	G =27 %	Intron	↑				

							compared with noncarriers (611)									
	rs2293152	x	-	✓	-	-	Major GG homozygotes for this SNP had increased risk of abdominal obesity compared with noncarriers (611)	G	C	G =38 %	Intron		↑			
	rs1053005	x	-	✓	-	-	Minor G allele carriers for this SNP had increased risk of abdominal obesity compared with noncarriers (611)	T	C	C = 20 %	UTR – 3	↑	↑			
TRAF6(606)	**rs331457**	x	-	✓	-	-	Reduced DNA repair and increased apoptotic rates (612)	C	T	T = 13 %	Intron					
ACSL5(607)	**rs2419621**	x	-	✓	-	-	Associated with marked weight loss in dieting and increased levels of ACSL mRNA in skeletal muscle	C	T	T = 26 %	nearGene-5		↓	↓		✓ (607, 613)

							biopsies(607, 613)									
FOXO1(286)	**rs2721068**	x	-	✓	-	-	A gain of function in FOXO1A in pancreatic β-cells resulted in decreased glucose use and decreased insulin secretion(614)	T	C	C = 34 %	Intron		↑	↓		
	rs17446614	x	-	✓	-	-	A gain of function in FOXO1A in pancreatic β-cells resulted in decreased glucose use and decreased insulin secretion(614)	G	A	A = 16 %	Intron		↑	↓		
	rs2701896	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	C	G =46 %	UK					
	rs17446593	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	G =14 %						

	rs17446593	x	-	✓	-	-	Minor allele carriers demonstrated lower insulin secretion(614). Previously explored for an association with cancer cachexia (203, 207)	A	G	G =17 %	UK		↑			✓ (203, 207)
	rs2721068	x	-	✓	-	-	Minor allele carriers demonstrated lower insulin secretion(614).	T	C	C = 48 %	UK		↑			
	rs17446614	x	-	✓	-	-	Minor allele carriers demonstrated lower insulin secretion(614).	G	A	A = 16 %	Intron		↑			
	rs2297627	x	-	✓	-	-	Minor allele carriers demonstrated lower insulin secretion(614).	G	A	G =50 %	Intron		↑			
FOXO3(286)	**rs2802292**	x	-	✓	-	-	Carriers of the minor G-allele of rs2802292 showed reduced fasting plasma insulin(615)	G	T	G =45 %	Intron		↓	↑		

	rs1935949	x	-	✓	-	-	Gain in function associated with increased life span(616)	A	G	A = 27 %	Intron					
	rs4946935	x	-	✓	-	-	Gain in function associated with increased life span(616)	A	G	A = 26 %	Intron					
	rs9486902	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 16 %						
IKKA(286) / CHUK	**rs11597086**	x	-	✓	-	-	Increased acute phase proteins and increase in plasma liver enzymes(617)	A	A/C	C = 22 %	Intron	↑				✓ (618)
	rs11591741	x	-	✓	-	-	*Reported to be associated with expression of its upstream gene CWF19L1 (CWF19-like 1) in human liver* (617) Marked response to anti TNF treatment, decrease of inflammation	G	C/G	C = 22 %	Intron	↓				✓ (617), (619)

							(619)									
MSTN(145, 286)	**rs1805086** 2379 A>G K153R	x	-	✓	-	-	Performed worse in skeletal muscle tests(620)	T	C	C = 6%	Missense (Lys - Arg)			↓		
	rs3791783	x	-	✓	-	-	Subjects with AA genotype have a much higher body weight, BMI, waist circumference, TC, TG and LDL-C than those with GG genotype (621)	C	T	C = 37 %	Intron		↑			
ACVR2B(145) / ACVR2B	**rs2268757**	x	-	✓	-	-	Carriers of ACVR2B Hap Group 1 exhibited significantly less quadriceps muscle strength than women homozygous for Hap Group 2(622)	C	T	T = 36 %	Intron			↓		
CPN1	**rs11597390**	x	-	✓	-	-	CPN1 encodes arginine carboxypeptidase-1, a liver-expressed	A	C/G /T	A = 25 %		↓				

							plasma metallo-protease that protects the body from potent vasoactive and inflammatory peptides containing C-terminal arginine or lysine (such as kinins or anaphylatoxins (617)									
TGFB2	**rs947712**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	T	C	T = 46 %	Intron					
	rs1890995	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 44 %	Intron					
	rs1418553	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	T = 48 %	Intron					

LTBP1	**rs817529**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	G =28 %	Intron					
SMAD2(145)							None									
SMAD3(145)							None									
TRIM63(623) / MuRF1							None									
FBXO32(79, 623) / Atrogin-1							None									
FBXO40(283)							None									
JunD(624)							None									
PLD1(625)							None									

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.8 Polymorphisms regulating adipose tissue biology

(4) Adipose tissue

The incidence of obesity and diabetes continues to increase worldwide. The obese patient is generating a new challenge in medicine and treatment. The physiology of obese patients differs remarkably from a normal weighted individual. Recently, patients suffering from advanced cancer have been found to be overweight rather than underweight (161). This has been shown to confound conventional measurements for risk stratification such as BMI. A recent study of pancreatic cancer patients has shown that severe muscle depletion when combined with obesity to be an independent adverse prognostic indicator in this patient group and should be considered as an alternative and more powerful means of risk stratification (12). It is however unclear how muscle depletion combined with overweight / obesity causes accelerated demise. The adipokines secreted by excess adipose tissue may act as systemic inflammatory mediators, inducing of insulin resistance in skeletal muscle and leading to a further increase in muscle protein loss. Increased lipolysis appears to be a key factor underlying fat loss, though decreases in lipid deposition and adipocyte development may also contribute (162). The following section examines polymorphisms in genes regulating adipose tissue metabolism.

(4a) Adipogenesis

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes (626). PPARα regulates key proteins involved in extracellular lipid metabolism, fatty acid oxidation and inflammation (627). PPARγ controls fat cell function and differentiation of new adipocytes and by inducing expression of genes promoting uptake of fatty acids, triglyceride synthesis and insulin sensitivity (628). Lipin proteins (lipin-1, lipin-2 and lipin-3) also act as transcriptional coactivators that regulate expression of lipid metabolism genes (629). SNPs in the above genes are shown in Table 5.12.

Table 5.12 - Summary of polymorphisms regulating adipogenesis

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
PPARA(193)	rs1800206 C484G	✓	-	-	✓	✓	Increased serum concentrations of total and LDL cholesterol (630) Increased serum IL-6 in 162Val homozygotes (631)	C	G	G = 2%	Missense 162 Leu→Val	↑	↑			
PPARG(193)	rs1801282 C49G	✓	-	-	✓	✓	Lower fasting insulin and insulin resistance (632, 633) Lower serum adiponectin levels (634, 635) Lower mRNA expression of PI3-kinase and higher PPARG	C	G	G = 7%	Missense 12 Pro→Ala		↑			✓

							expression in men (636)									
	rs3856806 C161T	✓	-	-	✓	✓	Lower fasting insulin and insulin resistance (637) Increased plasma leptin (638)	C	T	T = 12 %	Cds – synon His - His		↑			
	rs1800571	✓	-	-	✓	x	Defective phosphorylation at 114Ser leading to reduced ability to promote adipocyte differentiation (639)	C	A		MIssense 115 Pro→Gln		↑			
LPIN1(193)	rs2716609 C181T	✓	-	-	x	-	Higher insulin levels with C allele (640)	C	T	C = 25 %	Intron		↓			
	rs2577262	✓	-	-	x	-							*			
LPIN2	rs3745012	✓	-	-	✓	✓										

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies
* A/A homozygotes associated with higher resting metabolic rate (640)

(4b) Lipid turnover and transport

The lipid metabolism protein apolipoprotein C-III (apoC-III) inhibits triglyceride hydrolysis. Genetic variants have been shown to have lower fasting and postprandial serum triglycerides, higher levels of HDL-cholesterol and lower levels of LDL-cholesterol (641). A genetic link between lipid metabolism and inflammation has been suggested by the association between variation in the APOE gene and plasma CRP. A variant in the LRRFIP1 gene, which has been implicated in TNFa expression has been shown to be associated with adiposity and inflammation (642).

A recent experiment in tumour bearing mice demonstrated during the early and intermediate phases of tumour growth and cachexia, food intake remained normal while plasma levels of proinflammatory cytokines and zinc-α2-glycoprotein rose. The investigators found that genetic ablation of adipose triglyceride lipase (ATGL) prevented an increase in lipolysis and the net mobilisation of adipose tissue associated with tumour growth. Unexpectedly, they also observed that skeletal-muscle mass was preserved and that activation of proteasomal-degradation and apoptotic pathways in muscle was averted. Ablation of hormone-sensitive lipase (HSL) had similar but weaker effects. Genetic variants in both these new genes were explored and are listed in Table 5.13.

Zinc-α-2-glycoprotein (ZAG), otherwise known as LMF, is involved in the specific mobilisation of adipose tissue, with increased oxidation of released fatty acids, possibly via induction of uncoupling protein (UCP) expression (643). ZAG isolated from the MAC16 murine tumour, or from the urine of patents with cancer cachexia, stimulates lipolysis directly through interaction with adenylate cyclase in a guanosine triphosphate (GTP)-dependent process (644, 645). A polymorphism associated with change of function is shown in Table 5.13.

Table 5.13 - Summary of polymorphisms regulating lipid turnover and transport

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
ADRB1	rs1801253	✓	-	-	✓	✓	Diminished adenylyl cyclase activity (646) Diminished β1-AR-G-protein interaction (647)	C	G	G =30 %	Missense 389 Arg→Gly		↓			✓
	rs1801252	✓	-	-	X	-	Higher basal and agonist-stimulated adenylyl cyclase activity (648) Higher desensitization to sustained stimulation (648)	G	A	G =17 %	Missense 49 Ser→Gly		↑			
ADRB2	rs1042711 C-47T	✓	-	-	✓	x	Greater β2-AR expression in -19Cys (649)	C	T	C = 24%	Missense -19 Cys→Arg		↓			
	rs1800888 C491T	✓	-	-	✓	x	Diminished β2-AR-G-protein	C	T	T < 1%	Missense 164					

							interaction (650) Decreased native adipocyte receptor function (651)				Thr→Ile					
	rs1042713 G46A	✓	-	-	✓	✓	16Gly associated with increased insulin resistance (652)	G	A	A = 47%	Missense 16 Gly→Arg		↓			✓
	rs1042714	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	C	G= 24%						
	rs1042717	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 28%	Cds-synon (Leu-Leu)					
	rs1042719	x	✓	-	✓	✓	Previously explored for an association with cancer	G	C	C = 36%	Cds-synon (Gly-Gly)					

							cachexia (203, 207)									
ADRB3	rs4994	✓	-	-	✓	x	Associated with lower lipolytic activity (653)	G	A	G =10 %	Missense 64 Trp→Arg		↑			✓
APOC3(641)	**rs10892151**	x	-	✓	-	-	rs10892151 A carriers evidenced markedly lower FTG and ppTG than non-carriers(641)	C	T	T = 3%	Intron		↓			
GNB3	rs5443 C825T	✓	-	-	✓	✓	Associated with increased intracellular signal transduction (654)	C	T	T = 48%	Cds-synon (Ser-Ser)		↑			
LPL	rs328	✓	-	-	✓	✓	Significantly lower IL-8 levels (655) Increased LPL activity (656, 657)	C	G	G =10 %	Stop-Gain 447 Ser - XXX	↓	↓			✓
	rs17411031	x	-	✓	-	-	Associated with a lower risk of CHD an increase in Apolipoprotein AI and	G	C	G =24 %	U/K		↓			

							HDL-cholesterol, and reduced triglycerides(658)									
	rs1800590	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	T	G =11 %	(5' UTR)					
	rs326	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	G = 35%	Intron					
FABP1(193)	rs1801273 A340G	✓	-	-	x	-	Higher baseline free fatty acid (659)	C	T	T =0.1 %	Missense 94 Thr→Ala		↓			
APOE(470)	**rs769449**	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	T/C/A	A = 8%	Intron	↑	↑			
	rs157580	x	-	✓	-	-	Highly associated with serum CRP levels(470)	A	G	G =37 %	U/K					

LRRFIP1(642)	**rs11680012**	x	-	✓	-	-	Higher CRP levels and higher BMI due to exonic splicing change in variant(642)	G	C	C = 4%	Missense Arg–Thr	↑	↑			

PNPLA2 / ATGL(103)	**rs121918259**	x	-	✓	-	-	Systemic accumulation of triglycerides in cytoplasmic droplets (660)	C	T	U/K	Missense 195 PRO-LEU		↑	↓		
	rs121918260	x	-	✓	-	-	Systemic accumulation of triglycerides in cytoplasmic droplets (660)	C	T	U/K	Nonsense Stop-Gain 289 Gln - XXX		↑	↓		
	rs139576982	x	-	✓	-	-	Markedly impaired catalytic activities resulting in lowered FFA circulation(661)	G	A	U/K	Missense ARG–GLY		↓			
	rs145999340	x	-	✓	-	-	Markedly impaired catalytic activities resulting in lowered FFA circulation(661)	G	A	U/K	Missense ARG - HIS		↓			

	rs143718036	x	-	✓	-	-	Markedly impaired catalytic activities resulting in lowered FFA circulation(661)	G	A	U/K	Missense ARG - IIE		↓			
LIPE(103) / HSL	**rs1206034**	x	-	✓	-	-	Decreased serum cholesterol (662)	G	A	A = 15%	Intron		↓			
AZGP1(663) / ZAG	**rs4215**	x	-	✓	-	-	GG genotype subjects in rs4215 site have an increased susceptibility to obesity when compared with the AA+AG genotype subjects (664) rs4215 was associated with total cholesterol and LDL-C but not with	G	A	A = 42%		↑	↑			✓ (664), (665)

							HDL-C or TG(665)									
ITGB5(666)							None									
LOXL2(666)							None									
MFAP4(666)							None									
ACTNI(666)							None									
ECM2(666)							None									
DPT(666)							None									
LTBP2(666)							None									
FBLNI(666)							None									
CLIP(666)							None									
SPTANI(666)							None									
COX8A(666)							None									
CYC1(666)							None									

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

(4c) Adipokines and adipokine receptors

Adipose tissue, like skeletal muscle is an active metabolic and endocrine organ. A number of inflammatory cytokines secreted by adipose tissue have been shown with varying effect to influence the development of diseases such as insulin resistance, diabetes, and cancer cachexia by acting on muscle and fat metabolism (667). Adipokines regulate appetite, energy expenditure, insulin sensitivity, and the inflammatory response all of which have an important role in the pathogenesis of cancer cachexia (668). The adipokine resistin effects substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al.* 2006). SNPs in the adipokine genes are shown in Table 5.14.

Adiponectin is secreted from adipose tissue and binds to a number of receptors including adiponectin receptors 1 and 2. Adiponectin is produced by the adipocyte and has been shown to decrease insulin resistance. Unlike other adipokines associated with chronic inflammation, adiponectin is inversely related to insulin resistance and BMI. It appears to have protective metabolic and anti-inflammatory properties (128).

The adipokine, leptin plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. Polymorphisms in genes coding for adiponectin, leptin and their respective receptors are given in Table 5.14.

Table 5.14 - Summary of adipokine and adipokine receptor polymorphisms

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
RETN	rs1862513 C-420G	✓	-	-	✓	✓	Increased plasma resistin (669, 670)	G	C	G =31 %	U/K	↑	↑			✓
	rs34124816	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	C	C = 6%	nearGene-5					
ADIPOQ	rs17300539 G-11391A	✓	-	-	✓	x	Lower adiponectin levels with -11391GG and with -11377G allele (671)	G	T/C/A	A = 4%	nearGene-5			↑		
	rs266729 C-11377G	✓	-	-	✓	✓	Lower adiponectin levels with -11391GG and with -11377G allele (671)	C	G	C = 25%	nearGene-5			↓		
	rs1501299 G276T	✓	-	-	✓	✓	Increased adiponectin levels with	G	T	T = 32%	Intron		↓			✓

							T/T genotype (672, 673) Increased adiponectin mRNA levels in visceral fat with T allele (674)									
	rs2241766 T45G	✓	-	-	✓	✓	Increased plasma adiponectin (675, 676)	T	G	G =14 %	Cds – synon Gly - Gly		↓	↑		✓
ADIPOR1	rs666089 G-8503A	✓	-	-	X	-	Lower insulin sensitivity (677)	T	C	C <1%	Intron					
ADIPOR2	rs16928751 G795A	✓	-	-	✓	✓	795A, 870A, 963T haplotype associated with higher adiponectin levels (678)	G	A	A = 10%	Cds – synon Gln - Gln		↓			
	rs35854772 C870A	✓	-	-	✓	✓	795A, 870A, 963T haplotype associated with higher adiponectin levels (678)	C	A	A = 10%	Cds – synon Ile - Ile					
	rs9805042 C963T	✓	-	-	X	-	795A, 870A, 963T haplotype associated with higher	C	T	T = 21%	Cds – synon Tyr - Tyr					

							adiponectin levels (678)									
	rs767870	x	-	✓	-	-	Increased ADIPOR2 protein in monocytes(679)	A	G	G =25 %	Intron	↑	↑			✓ (680), (681)
LEP(193)	rs7799039 G-2548A	✓	-	-	✓	✓	Higher leptin levels (682)	G	A	A = 43%			↓			✓
LEPR	rs1137101 A27265G	✓	-	-	✓	✓	Higher insulin, leptin levels with 223Gln allele (683)	A	T/C/G	A = 41%	Missense 223 Gln→Arg		↓			✓
	rs1137100 A5193G	✓	-	-	✓	✓	Decreased fasting insulin levels in postmenopausal women with impaired glucose tolerance (684) Increased leptin levels in 109Lys homozygotes (685)	A	G	G =38 %	Missense 109 Lys→Arg		↓			✓
	rs8179183 G44704C	✓	-	-	✓	✓	Decreased leptin levels in obese patients following	G	C	C = 15%	Missense 656 Lys→Asn		↑			

							lifestyle modification in 656Lys homozygotes (686) Increased fasting insulin levels in postmenopausal women with impaired glucose tolerance (684)									
	rs3790419															
	rs1805134	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 19%	Cds – synon Ser - Ser					
	rs1892534	x	-	✓	-	-	Highly associated with serum CRP levels(470)	T	C	C = 45%	Rev	↑				✓ (687)
	rs12753193	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	A	A = 46%	UK	↑				

	rs7539471	x	-	✓	-	-	Highly associated with serum CRP levels(470)	G	T/C/A	A = 19%	UK	↑				
	rs12409877	x	-	✓	-	-	Highly associated with serum CRP levels(470)	A	G	G =41 %	Intron	↑				
	rs5010905	x	-	✓	-	-	Highly associated with serum CRP levels(470)	C	T	C = 21%	Uk	↑				

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.9 Polymorphisms regulating appetite

(5) Appetite

Muscle mass is clearly sensitive to food intake. The pathogenesis of cancer anorexia is multifactorial and reflects the complexity of the mechanisms controlling energy homeostasis under physiological conditions. The main molecular mechanisms regulating the cancer anorexia-cachexia syndrome include alterations in brain neurochemistry. In particular, the hypothalamic melanocortin system appears not to respond appropriately to peripheral inputs, and its activity is diverted largely toward the promotion of catabolic stimuli promoting metabolism of carbohydrates, lipids, and proteins in peripheral tissues leading to insulin resistance, increased lipolysis, and accelerated muscle proteolysis (13). Ghrelin is produced by the P/D1 cells of the stomach and acts as the natural counterpart to leptin. Ghrelin exerts its effects by promoting food intake (via the orexigenic NPY system) (688) and decreases sympathetic nerve activity (689). G-protein coupled melanocortin receptors bind *α*-melanocyte stimulating hormone (*α*-MSH). Melanocortin 3 and 4 receptors are involved in feeding behaviour and the regulation of metabolism (690).

Neuropeptide Y (NPY) acts as a neurotransmitter in the brain and in the autonomic nervous system. An increase in NPY signalling leads to increased food intake (691). Genetic variants have been added to this new gene and are listed in Table 5.15.

Table 5.15 - Summary of polymorphisms regulating appetite

Gene	SNP	Old S/R	Gen	New S/R	Used	Vali d-ated	Functional Significance	Ances tr-al allele	SNP allele / s	MAF	SNP Type	Systemic Inflamm a-tion	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
GHRL(193)	rs696217 C247A	✓	-	-	✓	✓	Lower insulin secretion during glucose tolerance test (692)	G	T	T = 10 %	MIssense 72 Leu→Met		↑			✓
	rs34911341 G346A	✓	-	-	x	-	51Gln allele associated with lower ghrelin concentrations (693)	C	T	T <1 %	Missense 51 Arg→Gln		↑			
	rs26802	✓	-	-	✓	x										
	rs42451	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 21 %	Intron					
	rs35681	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 33 %	Intron					
MC3R(694)	**rs3746619 C17A**	x	-	✓	-	-	Related with in vitro diminished functionality and expression of the receptor, showing a	A	C	A = 21 %	Missense 6 Thr-Lys					

							significant association with childhood obesity in a case-control study(694)									
	rs3827103 G241A	x	-	✓	-	-	Related with in vitro diminished functionality and expression of the receptor, showing a significant association with childhood obesity in a case-control study(694) Reduced signaling activity compared with wild-type MC3R(695)	G	A	A = 20 %	Missense 81 Val-Ile		↑			✓ (694), (695)
MC4R	rs2229616 G307A	✓	-	-	✓	x	Lower serum triglyceride levels (696)	C	T	T = 2%	MIssense 103 Val→ Ile		↓			✓
	rs52820871	✓	-	-	✓	x										
	rs17782313	x	-	✓	-	-	Near MC4R variants appear to contribute to body fat, body fat distribution,	T	A/C/G	C = 22 %	UK		↑			

							some metabolic traits, weight Development (697)									
	rs17700633	x	-	✓	-	-	Near MC4R variants appear to contribute to body fat, body fat distribution, some metabolic traits, weight Development (697)	A	G	A = 20 %	UK		↑			
CNR1	rs1049353	✓	-	-	✓	✓										
Npy(581)	**rs16147**	x	-	✓	-	-	C-allele has strongly reduced affinity for a yet unknown factor compared to the T-allele and leads to increased gene expression(698)	T	C	T = 49 %	nearGene-5	↑				✓ (699)
	rs16139	x	-	✓	-	-	leucine7 to proline7 in the signal peptide of preproNPY has been associated with increased risk factor for many cardiovascular diseases, such as accelerated	T	C	C = 6%	Missense Leu – Pro	↑	↑			✓ (701), (702).

							atherosclerosis(700). Furthermore, elevated serum lipid levels have been associated with the P7 allele in certain populations(701). Additionally, the L7P polymorphism has been shown to increase the risk of type II diabetes (T2D) as well as earlier onset of the disease and vascular complications(702).									
Ang II(581)							None									

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.4.10 Other notable polymorphisms

(6) Others

Metallothionein (MT) are low molecular weight proteins enriched with cysteine. MTs have the capacity to bind both physiological (such as zinc, copper, selenium) and xenobiotic (such as cadmium, mercury, silver, arsenic) heavy metals through the thiol group of its cysteine residues, which represents nearly the 30% of its amino acidic residues. Zinc homeostasis is often disrupted in cancer cachexia. It has been proposed that the acute phase response can mediate zinc redistribution and accumulation in skeletal muscle tissue and contribute to the activity of the UPP that regulates protein catabolism (703).

The P2Y-receptors mediate the actions of extracellular nucleotides in cell-to-cell signalling. The P2Y11 receptor is highly expressed in immunocytes and may play a role in the differentiation of these cells (704). SNPs in these genes are listed in Table 5.16.

A number of new genes and SNPs are listed in Table 5.16, these have evolved from a recent candidate gene approach study identifying SNPs in cancer cachexia. Target SNPs identified from the previous review which were unable to be genotyped in a cancer population due to experimental design were substituted for the nearest SNP with a linkage disequilibrium of >0.9. These SNPs have been added to the current review as they have been genotyped in patients with cancer cachexia.

Table 5.16 - Summary of other notable polymorphisms

Gene	SNP	Old S/R	Gen	New S/R	Used	Validated	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Cancer Survival	Repeat studies
MT1B	rs1875233	✓	-	-	✓	✓										
MT2A	rs1610216 A-209G	✓	-	-	x	-	A/A genotype associated with higher plasma levels of IL-6 (705)	G	A	G =39%	UK	↓				
	rs34326929	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	A	A <1%	nearGene-5					
	rs10636 C838G	✓	-	-	✓	✓	C allele associated with increased MCP-1 and decreas	C	G	C = 24%	(3' UTR)	↑				✓

							ed NK cell cytotoxicity (706)									
P2RY11	rs3745601	✓	-	-	x	-	Elevated levels of CRP (707)	G	A	A = 18%	Missense 87 Ala→Thr	↑				
	rs12460842	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 39%	Intron					
TAF12(203, 207)	**rs3795845**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	T	G	T = 27%	Intron					
	rs1804642	x	✓	-	✓	x	Previously explored for an association with	C	G	?	Missense Met - Ile					

							cancer cachexia (203, 207)									
CXCR7(203, 207)	**rs10183022**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 40%	Intron					
	rs9287599	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	G =12%	Intron					
	rs1045879	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 28%	Cds Synon Leu – Leu					

KBTBD5(203, 207)	**rs6805421**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	A = 37%	Missense Asn - Ser					
	rs123509	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	T	C	T = 23%	Missense Cys – Arg					
	rs3846062	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	C	A = 43%	UTR - 3					
APEH(203, 207)	**rs4855881**	x	✓	-	✓	✓	Previously explored for an associati	G	A	G =46%	Intron					

							on with cancer cachexia (203, 207)									
	rs2960548	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	G	C = 39%	Intron					
LY6G5B(203, 207)	**rs2142234**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 4%	Intron					
	rs9267532	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 4%	Missense Arg - Cys					

	rs1266076	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	G	G =31%	nearGene-3					
CAMK2B(203, 207)	rs917791	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	G=29 %	Intron					
	rs10441113	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A/T/C	A=24 %	Intron					
	rs4526269	x	✓	-	✓	x	Previously explored for an associati	G	T	T=21 %	Intron					

							on with cancer cachexia (203, 207)									
DCTN3(203, 207)	**rs3802427**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T=23 %	Intron					
TTC18(203, 207)	**rs4294502**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 7%	Missense Asn - Asp					
	rs3812621	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	C = 10%	Intron					

NUP160(203, 207)	**rs11039426**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 34%	Intron				
HYLS1(203, 207)	**rs3088241**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	G	C = 43%	Missense Glu - Asp				
	rs549990	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	A	C = 28%	Intron				
	rs622756	x	✓	-	✓	✓	Previously explored for an associati	A	C	A = 10%	Intron				

							on with cancer cachexia (203, 207)									
	rs547232	x	✓	-	✓	✓	Different gene DDX25 PUS3	T	C	T=37 %	Intron					
DCD(203, 207)	**rs2029851**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	C = 33%	Intron					
FARP1(203, 207)	**rs3848017**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 34%	Intron					
	rs584800	x	✓	-	✓	✓	Previously explored for an association with	C	T	T = 15%	Intron					

							cancer cachexia (203, 207)									
GPS1(203, 207)	**rs4969484**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	T	T = 49%	Intron					
APCDD1(203, 207)	**rs3748415**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	A = 13%	Missense Val - Ile					
EIF3G(203, 207)	**rs3826785**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 20%	Intron					

GCDH(203, 207)	**rs11085824**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	G =28%	nearGene-5					
	rs9384	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	T	T = 8%	UTR - 3					
TH1L(203, 207)	**rs163781**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	A	G=46 %	Intron					
CTSZ(203, 207)	**rs163792**	x	✓	-	✓	✓	Previously explored for an associati	C	A	A=46 %	Intron					

							on with cancer cachexia (203, 207)									
TFRC[85 86]	**rs2284890**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 37%	Intron					
	rs41301381	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	G	C	C = 1%	Missense Leu - Val					
	rs3817672	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 34%	Missense Gly - Ser					

	rs9877119	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	A	G	G= 37%	Intron					
ZER1	**rs13284665**	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	A	G	G = 9%	Intron					
	rs8507	x	✓	-	✓	x	Previously explored for an association with cancer cachexia (203, 207)	G	T	T = 4%	Cds Synon Ala – Ala					
	rs4836625	x	✓	-	✓	x	Previously explored for an associati	T	C	C = 32%	Intron					

							on with cancer cachexia (203, 207)									
TSC2	**rs7187438**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	T	C	C = 37%	Intron					
FOXC2	**rs34221221**	x	✓	-	✓	✓	Previously explored for an association with cancer cachexia (203, 207)	C	T	T = 44%	nearGene-5					

↑ Increase; ↓ Decrease; ↕ Increase or decrease (evidence conflicting); ✓ Presence of repeat studies

5.5 Analysis of results

5.5.1 Clinical significance

Clinical significance was defined as any SNP affecting more than one of the recognised hall marks of cancer cachexia. Inflammation has been shown to influence the severity of cancer cachexia and was therefore identified as a clinical feature. Changes in body composition of muscle and/or fat mass form the basis of cancer cachexia, therefore SNPs that have the potential to alter an individual's body composition, whether to increase or decrease these components will play a significant clinical role in the development of cancer cachexia or not. Lastly, any SNPs influencing overall survival were included in a definition of clinical significance.

Out of 281 candidate polymorphisms that were identified and summarised here, the functional or clinical significance of 80 polymorphisms have been verified in more than one study. Of these 80 polymorphisms, 24 have been shown to have more than one association with clinical features associated with cancer cachexia (i.e. inflammation, changes in lean and/or fat mass, and overall survival), these are termed promising SNPs. An in-depth analysis of the 24 promising SNPs as biomarkers for susceptibility of cancer cachexia (Table 5.17) is presented below. Thirteen of these are new promising SNPs and eleven of these SNPs have been described previously as promising SNPs (193) but have been included for completeness.

(1, 2) The G allele of TLR-1 (-7202A/G) (rs5743551) is associated with elevated TLR1-mediated cytokine production (439, 441). TLR1 (-7202G) marks a coding SNP that causes higher TLR1-induced NF-kB activation and higher cell surface TLR1 expression (441). Toll-like receptor (TLR) pathways are critical components of the immune response to pathogens and disease (708). This particular polymorphism has been shown to lead to decreased survival in patients with sepsis (441) and NSCLC (440). In addition to this SNP in TLR-1, patients carrying the mutant allele T of TLR4 1196C/T (Thr399Ile, rs4986791) had lower TNF-α and sTNFR2 levels compared to patients carrying wild-type alleles (446). These patients carrying the mutant phenotype have also been shown to have increased total body fat, visceral fat, liver fat and decreased insulin sensitivity (448).

(3) Steps in the inflammatory process include accumulation of lipids, recruitment of leukocytes and smooth muscle cells into vessel walls, and accumulation of

extracellular matrix. Intercellular adhesion molecule-1 (ICAM-1) is integral in these cellular processes as interactions between ICAM-1 and activated receptors on the leukocytes result in firm adhesion and transmigration of leukocytes into the basement membrane of the vasculature. The T-allele of rs5491 encodes a lysine to methionine substitution in exon 2 in the N-terminal domain of ICAM-1 and results in a protein that is unable to bind to fibrinogen and has a decreased affinity for T-cells at lower ICAM-1 concentrations compared to wild type ICAM-1 (709). This leads to increased circulating levels of sICAM-1 and an association with insulin resistance and the metabolic syndrome (461).

(4) The C allele of the A37674C *SELP* polymorphism (rs6136) is associated with decreased serum P-selectin levels (462, 463). Localisation of neutrophils in acute inflammation and of macrophages in later stages of the inflammatory response are dependent on P-Selectin and serum levels of P-selectin have been found to be significant prognostic factors in survival in patients with gastric and colorectal malignancies (710, 711). Patients with cancer who carry the C-allele of the rs6136 polymorphism in *SELP* gene are at reduced risk of developing cachexia as defined by weight loss >10% (207).

(5-7) The cytokine TNF-*α* precipitates systemic inflammation via the acute phase reaction. The –308A allele (rs1800629) has been associated with an increased TNF-*α* production as well as a 6-fold increase in transcription of the *TNF* gene (471, 472). Women carrying the A/A genotype have been linked to increased fat accumulation (712). The -863A allele (rs1800630) associated with decreased transcriptional activity and reduced serum TNF-*α* levels (473, 474, 476, 477). Obese people express 2.5-fold more TNF mRNA in fat tissue (115). The -238A allele of the SNP rs361525 has shown decreased transcriptional activity of TNFα (473), as well as decreased peripheral mononuclear blood cells (PMBC) production of TNF-α after stimulation with T-cell mitogens (473). It has also been shown to decrease insulin resistance (474).

(8) LTA is part of the tumour necrosis factor family, it is produced by lymphocytes, and mediates inflammatory responses, stimulation of immune system, and apoptosis (713). The G allele of the 252 A>G polymorphism (rs909253) has been associated with increased serum TNF-*α* levels (479, 480), and patients who are A/A homozygotes have been linked with better prognosis in lung cancer and gastric cancer (714, 715).

(9 - 11) IL-1β is a product of the *IL1B* gene and plays a role in potentiating an inflammatory response. The -31 C>T (rs1143627) and -511 C>T (rs16944) polymorphisms in the promoter region of the *IL1B* gene have been linked with increased transcriptional activity of the *IL1B* gene and subsequently increased IL-1β production (484). These two alleles are also linked with poorer progression-free survival and overall survival in advanced gastric cancer (716). A synonymous C to T polymorphism at nucleotide position 3953 (rs1143634) has resulted in Increased IL-1β levels (487). The T/T genotype has also been associated with lower plasma levels of IL-1 receptor antagonist (IL-1RA) (486). In addition, the T allele has found to be a major risk factor for cachexia in gastric cancer (209), as well as being linked to lower total fat mass (717). The T/T genotype was found to be associated with shorter survival in pancreatic cancer (718). These three SNPs may be potential candidates for cancer cachexia.

(12) IL-6 mediates B-cell differentiation and maturation, immunoglobulin secretion, cytotoxic T-cell differentiation and acute-phase protein production (719). The -174 G>C promoter polymorphism (rs1800795) in the *IL6* gene has been associated with lower serum levels of IL-6(490). The G allele has been linked to higher fasting insulin and lower adiponectin levels which may have a role in the regulation of adiposity (491). In addition the C/C genotype has been associated with lower fat free mass and increased waist circumference (675, 720).

(13) IL-18 is produced by macrophages and functions by binding to the interleukin-18 receptor inducing cell-mediated immunity following infection. After stimulation with IL-18, natural killer (NK) cells and certain T cells release interferon-γ (IFN-γ) that plays an important role in activating the macrophages. Apart from its physiological role, IL-18 is also able to induce severe inflammatory reactions. Individuals with the allele 105AA (rs549908) demonstrated increased IL-18 production from LPS and A23187 + PMA stimulated monocytes (499). Haplotype of common alleles have been shown to be associated with significantly lower IL-18 (501).

(14) IGF-1 is one of the most potent natural activators of the AKT signalling pathway which is the main stimulator of cell growth and multiplication. IGF-1 also mediates many of the growth-promoting effects of growth hormone (GH) (542). The genotype CC of rs7136446 associated with higher body fat and increased maximal force production (545), it has also been shown to be significantly associated with elevated levels of circulating IGF-I (543).

(15) The glucocorticoid receptor (GR, or GCR) also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1) is the receptor to which cortisol and other glucocorticoids bind. In the absence of glucocorticoids, the glucocorticoid receptor (GR) resides in the cytosol complexed with a variety of proteins including heat shock protein 90 (hsp90), heat shock protein 70 (hsp70) and the protein FKBP52 (FK506-binding protein 52) (721). The endogenous glucocorticoid hormone cortisol diffuses through the cell membrane into the cytoplasm and binds to the glucocorticoid receptor (GR) resulting in release of the heat shock proteins. Activated GR can bind to the transcription factor NF-κB and prevent it from up regulating target genes (722). The mutant allele of the N363S (rs6195) SNP enhances glucocorticoid sensitivity by increasing gene transcription (560). Indeed, in various studies, a link was established between the N363S SNP and characteristics of a Cushingoid phenotype, including increased BMI and waist circumference, dyslipidaemia and augmented fasting insulin levels, indicating reduced insulin sensitivity (723, 724).

(16) The glucokinase regulatory protein (GKRP) also known as glucokinase (hexokinase 4) regulator (GCKR) is a protein produced in hepatocytes. GKRP binds glucokinase (GK), thereby controlling both activity and intracellular location of this key enzyme of glucose metabolism (558, 725). The glucose-increasing major C allele of rs780094 of GCKR has been shown to be significantly associated with increased insulin resistance leading to development of T2DM and altered lipid metabolism (569, 726).

(17) CNTF is involved in the neuroendocrine signalling of appetite. It leads to marked weight loss through suppressed food intake without causing hunger or stress (727). CNTF receptor-α is abundantly expressed in skeletal muscle (728, 729). As such, recent studies have examined the roles of CNTF and CNTF genotype on neuromuscular disease and muscle function. CNTF administration has been shown to prevent losses of soleus muscle mass and function after hindlimb suspension in rats (729). In humans, the A allele mutation of rs1800169 possess significantly greater muscular strength and muscle quality at relatively fast contraction speeds than the ancestral G alelle individual (586). This polymorphism has also been associated with a global weight gain in healthy humans (587).

(18) The uptake of FFA by skeletal muscle for metabolism is initiated by transmembrane acyl-CoA synthetase long-chain (ACSL) proteins that esterify FFAs to acyl-coenzyme A (acyl-CoA) molecules. Acyl-CoA species are used mainly in both the synthesis of cellular lipids and the degradation of fatty acids *via* β-oxidation. Small increases in the expression of ACSL5 in skeletal muscle could have profound effects on FFA utilisation (613). A strong association between the common SNP rs2419621 and rapid weight loss in obese Caucasian females in response to restricted diet has been demonstrated (607). The SNP located 12 nucleotides upstream of the second transcription start site of the ACSL5 gene is characterized by a cytosine [rs2419621(C)] to thymine [rs2419621(T)] transition. This study also demonstrated that the T allele is associated with a 2.2-fold increase of ACSL5 transcript level in skeletal muscle biopsies when compared to noncarriers (607). In a further study T allele variants were shown to create a functional cis-regulatory E-box element (CANNTG) that is recognised by the myogenic regulatory factor MyoD. The T allele promoted MyoD-dependent activation of a 1089-base pair ACSL5 promoter fragment in non-muscle CV1 cells. Differentiation of skeletal myoblasts significantly elevated expression of the ACSL5 promoter. The T allele variants sustained promoter activity 48 h after differentiation, whereas the C allele variants showed a significant decline. These results revealed a mechanism for elevated transcription of ACSL5 in skeletal muscle of carriers of the rs2419621 (T) allele, associated with more rapid diet-induced weight loss. This is the first example of a MyoD binding polymorphism conferring differential promoter activity of a metabolic gene (613).

(19) Lipoprotein lipase (LPL) plays a central role in the overall lipid metabolism and transport (730). The rs328 polymorphism in the *LPL* gene leads to a premature stop codon at amino acid 447. The stop codon results in lower LPL activity (656, 657), and is associated with lower levels of IL-8 (655). Individuals not in possession of the stop codon are associated with central obesity (731).

(20) Zinc-α-2-glycoprotein (ZAG), otherwise known as LMF, is involved in the specific mobilisation of adipose tissue, with increased oxidation of released fatty acids, possibly via induction of uncoupling protein (UCP) expression (643). LMF isolated from the MAC16 murine tumour, or from the urine of patents with cancer cachexia, stimulated lipolysis directly through interaction with adenylate cyclase in a guanosine triphosphate (GTP) dependent process (644, 645). This effect was also produced by the interaction of LMF with the β_3-adrenoceptor (732). Genotypes of

rs4215 in ZAG gene have been suggested to be significantly associated with obesity. The GG genotype subjects in rs4215 site have an increased susceptibility to obesity when compared with the AA+AG genotype subjects (664). In a separate study variations in the rs4215 genotype have been linked with changes in circulating levels of total cholesterol and LDL-C.

(21) The adipokine resistin potentiates a pro inflammatory state, resistin also appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways(733). The –420 C>G polymorphism (rs1862513) is shown to be linked to increased plasma resistin (669, 670), and individuals with the G/G genotype are associated with an increased prevalence of obesity (734). Overall, increased plasma resistin has to shown to correlate with increased CRP and insulin resistance (669, 735-738).

(22, 23) Adiponectin is a protein hormone that is exclusively secreted from adipose tissue and modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism (739). The *ADIPOQ* gene, which codes for adiponectin, has a 45 T>G polymorphism (rs2241766) that is associated with increased plasma adiponectin (675, 676). Individuals with G/G genotype have been observed to be leaner with less abdominal fat (740). The adiponectin receptors ADIPOR2, serves as a receptor for adiponectin and mediates increased AMPK and PPAR-α ligand activities, as well as fatty acid oxidation and glucose uptake by adiponectin(741). In peripheral monocytes, carriers of the major A allele (homozygotes and heterozygotes) of rs767870 polymorphism had higher levels of ADIPOR2 protein expression compared to homozygotes of the minor G allele (679). This same SNP has also been associated with liver fat content and the incidence of type II DM (680, 681).

(24) Pro-inflammatory cytokines (TNFα and IL-1β) and hypothalamic serotonergic neurons have been implicated in the dysfunction of the hypothalamic melanocortin system (29). The orexigenic neuropeptide Y (NPY) peptide system appears to be strongly influential in the control of feeding (30). The pathway originates in the hypothalamic arcuate nucleus (ARC) and extend projections widely over the brain (30). The role of cytokines in cancer anorexia may be affected through influence on the NPY system. The genetic variant rs16139 causing, leucine7 to proline7 in the signal peptide of preproNPY has been associated with increased risk factor for many cardiovascular diseases, such as accelerated atherosclerosis (700). Furthermore,

elevated serum lipid levels have been associated with the P7 allele in certain populations (701). Additionally, the L7P polymorphism has been shown to increase the risk of type II diabetes as well as earlier onset of the disease and vascular complications (702).

SNP number Gene	SNP	Previous S/R	New S/R	Functional Significance	Ancestral allele	SNP allele / s	MAF	SNP Type	Systemic Inflammation	BMI/Fat mass	Lean mass /Strength	Survival	Repeat studies
1 TLR-1	rs5743551		✓	The G allele of TLR-1 (-7202A/G) (rs5743551) is associated with elevated TLR1-mediated cytokine production (441) Alleles -7202G and 248Ser, and the 248Ser-602Ile haplotype were associated with circulatory dysfunction among severe septic patients (0.001 ≤ p ≤ 0.022), and with reduced IL-10 (0.012 ≤ p ≤ 0.047) and elevated CRP (0.011 ≤ p ≤ 0.036) serum levels during the first week of sepsis	C	T	T = 47%	nearGene-5	↑(439)			↓ (440)	✓(439), (441), (440)

				development (439).									
2 TLR-4	rs4986791		✓	Serum levels of TNF-alpha and its soluble receptors are elevated and associated with increasing BMI values in obese children. Serum cytokine levels, as modifying factors of insulin resistance, may be affected by TLR4 polymorphisms in obese children (446).	C	T	T = 3%	Missense Thr - Ile	↑(447)	↑ (448)			✓ (449)
3 ICAM-1	rs5491		✓	ICAM1 DNA segment variants were associated with sICAM-1 protein level including the novel finding that levels differ by the functional variant rs5491 (460)	A	T	T = 7%	Missense Lys - Met	↑	↑ (461)			✓ (461)

4 SELP	rs6136	✓		Decreased serum P-selectin levels (462, 463) P-selectin genotype is associated with the development of cancer cachexia (207).	T	G	G=4 %	715 Missense Thr – Pro	↓(462, 463)			↑	✓(207), (462, 463)
5-7 TNF-α	rs1800629	✓		Increased TNF-α production (471) Six fold increase in transcription of TNF-α (472)	G	A	A = 10%	nearGene-5	↑	↑			✓ (472), (471)
	rs361525		✓	Decreased transcriptional activity (473) Decreased PMBC production of TNF-α after stimulation with T-cell mitogens (473) Decreased insulin resistance (474)	G	A	A = 5%	nearGene-5	↓	↓			✓

	rs1800630	✓		Reduced total serum IgE levels (476) Reduced serum TNF-α levels (476) 31 % decrease in transcription of TNF-α (477)	C	A	A = 15%	nearGene-5	↓	↓			✓
8 LTA	rs909253	✓		Increased serum TNF-α levels (479, 480)	A	G	G =40 %	Intron	↑			↓	✓
9-11 IL-1β	rs1143627	✓		Increased expression of IL-1β gene with T allele (483) Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (484) Increased transcriptional activity with -31T/-511C/-1470G haplotype (484)	G	A	G =48 %	nearGene-5	↑			↑	✓
	rs16944	✓		Increased IL-1β production from whole blood	A	G	A = 48%	nearGene-5					

				leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (484) Increased transcriptional activity with -31T/-511C/-1470G haplotype (484) No significant increase in IL-1β production in response to LPS in patients homozygous for T allele (485)					↓			↓	✓
	rs1143634	✓		T/T genotype associated with lower plasma levels of IL1-RA (486) Increased human amniochorion IL-1β production after stimulation with LPS (487)	G	A	A = 15%	Synonymous Phe - Phe	↑	↓		↓	✓
12 IL-6	rs1800795	✓		Lower levels of IL-6 in plasma in healthy subjects (490)	G	C	C = 19%	nearGene - 5	↓	↑	↓		✓

				Higher fasting plasma insulin levels with G allele (491) Lower circulating adiponectin levels with G allele (491)									
13 IL-18	rs549908		✓	Increased IL-18 production from LPS and A23187 + PMA stimulated monocytes in 105AA and -137GG (499) Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (501)	T	G	G =23 %	Synonymo us Ser - Ser	↑	↓			✓ (499), (501)
14 IGF-1	rs713644 6		✓	Genotype CC of rs7136446 associated with higher body fat and increased maximal force production(545) Significantly associated with elevated levels of IGF-I (543)	T	C	C = 29%	Intron	↑	↑	↑		✓ (543),(545),

15 NR3C1	rs6195		✓	Reduced first-phase glucose-stimulated insulin secretion and disposition index in women, but not in men (565). Associated with enhanced glucocorticoid sensitivity (560).	A	G	G = 5%	Missense Asn – Ser	↓	↓			✓ (565), (566)
16 GCKR	rs780094		✓	Highly associated with serum CRP levels (470)	C	T	T = 39%	Intron	↑	↑			✓ (470), (569)
17 CNTF	rs1800169		✓	G/A genotype possess significantly greater muscular strength and muscle quality at relatively fast contraction speeds than do G/G individual (586)	G	A	A = 12%	Intron		↑	↑		✓ (586), (587)
18 ACSL5	rs2419621		✓	Associated with marked weight loss in dieting and increased levels of ACSL	C	T	T = 26%	nearGene-5		↓	↓		✓ (607, 613)

				mRNA in skeletal muscle biopsies (607, 613)									
19 LPL	rs328	✓		Significantly lower IL-8 levels (655) Increased LPL activity (656, 657)	C	G	G =10 %	Stop-Gain 447 Ser - XXX	↓	↓			✓
20 ZAG	rs4215		✓	GG genotype subjects in rs4215 site have an increased susceptibility to obesity when compared with the AA+AG genotype subjects (664) rs4215 was associated with total cholesterol and LDL-C but not with HDL-C or TG (665)	G	A	A = 42%		↑	↑			✓ (664), (665)
21 RETN	rs186251 3	✓		Increased plasma resistin (669, 670)	G	C	G =31 %	U/K	↑	↑			✓
22 ADIPOQ	rs224176 6	✓		Increased plasma adiponectin (675, 676)	T	G	G =14 %	Cds – synon Gly - Gly		↓	↑		✓

23 ADIPOR 2	rs767870		✓	Increased ADIPOR2 protein in monocytes (679)	A	G	G =25 %	Intron	↑	↑			✓ (680), (681)
24 NPY	rs16139		✓	leucine7 to proline7 in the signal peptide of preproNPY has been associated with increased risk factor for many cardiovascular diseases, such as accelerated atherosclerosis (700). Furthermore, elevated serum lipid levels have been associated with the P7 allele in certain populations (701). Additionally, the L7P polymorphism has been shown to increase the risk of type II diabetes (T2D)	T	C	C = 6%	Missense Leu – Pro	↑	↑			✓ (701), (702).

				as well as earlier onset of the disease and vascular complications (702).									

Table 5.17 - Polymorphisms replicated in more than one study and with at least two effects on clinical features associated with cancer cachexia (n = 24)

5.5.2 Pathway analysis

The 80 polymorphisms which have been verified in more than one study were found across 51 genes. These genes were entered into the IPA algorithm as focus genes and were found to be significantly interconnected in two major networks (Table 5.18). The two networks are presented in Figures 5.2 and 5.3.

Network	Molecules*	Calculated score	Focus genes	Top functions of network
1	ADCY, **ADRB1, ADRB2, ADRB3**, Beta arrestin**, CCL2, CRHR1**, Cytochrome c, Endothelin, estrogen receptor, G protein, G protein alphai, G Protein β, GNRH, Gpcr, Gs-coupled receptor, **IGF1R,** IL-17f dimer, **IL-6R,** Jnk, **LEP, MC3R, MC4R,** Mmp, NADPH oxidase, **NPY,** P110, p85 (pik3r), Pdgfr, PLC, Shc, **TRHR,** trypsin, TSH, Voltage gated calcium channel	22	12	Cell signalling, Neurological disease, Nutritional disease
2	3 BETA HSD, **ADIPOQ, ADIPOR2,** Akt, AMPK, **AZGP1**, **FOXO1, GHRL,** Gm-csf, HLA-DQ, Igf, **IGFBP3,** IL-8r, jINK1/2, MTORC1, Na+, K+ - ATPase, Nr1h, Pde4, PEPCK, **PIK3CB,** Ppp2c, PRKAA, Proinsulin, Ptk, Rab5, Rxr, Scavenger receptor class A, sPla2, T3-TR-RXR, thymidine kinase, **UCP2, UCP3, VDR,** VitaminD3-VDR-RXR	20	10	Endocrine System, Development and Function, Carbohydrate Metabolism, Molecular Transport

Table 5.18 - Ingenuity pathway analysis of genes that were replicated in more than one study (n = 51). *Focus genes in bold. The software illustrates networks graphically and calculates a score for each network, which represents the approximate 'fit' between the eligible focus molecules and each network. The network score is based on the hypergeometric distribution and is reported as the –log (Fisher's exact test result).

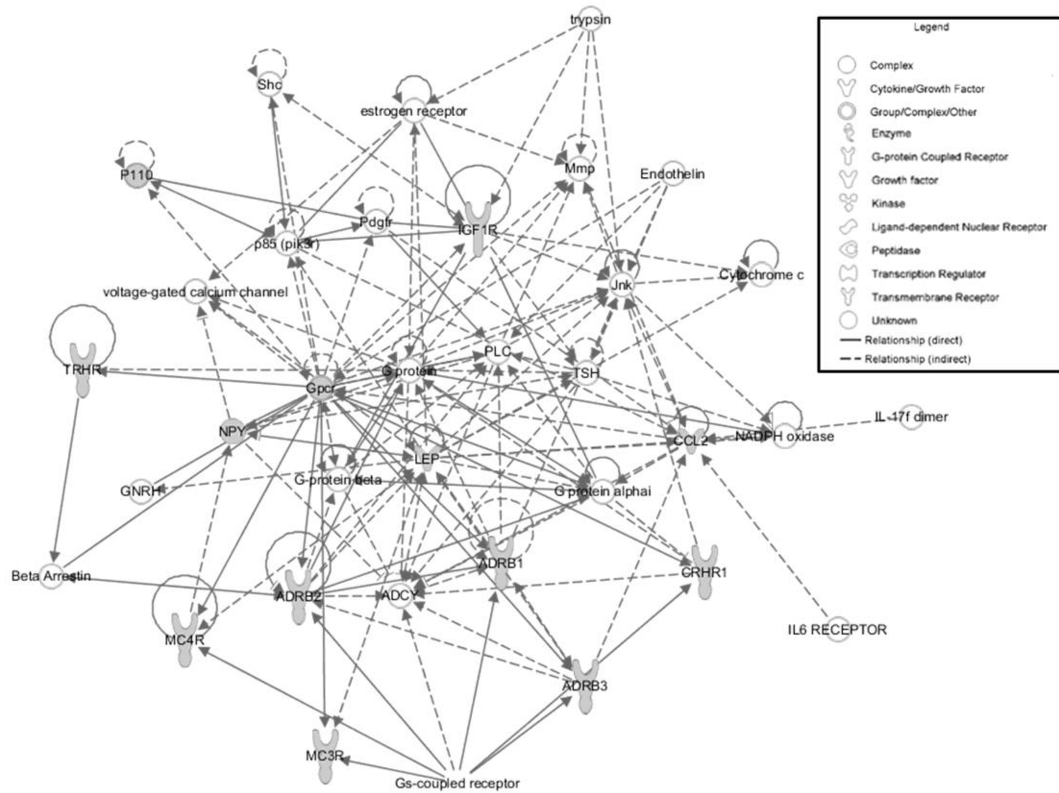


Figure 5.2 - Connection map for first ranked network. Genes with variants that had functional or clinical associations replicated in at least one study were entered into the ingenuity pathway analysis software for an unsupervised functional analysis to discern regulatory networks that involved these molecules. Focus genes are shaded in grey. Solid lines show direct interaction (binding/physical contact); dashed lines show indirect interaction that is supported by the literature but possibly involving ≥1 intermediate molecules that have not been investigated definitively. Molecular interactions that involved only binding are connected with a line without an arrowhead because directionality cannot be inferred.

Figure 5.3 - Connection map for second ranked network. Genes with variants that had functional or clinical associations replicated in at least one study were entered into the ingenuity pathway analysis software for an unsupervised functional analysis to discern regulatory networks that involved these molecules. Focus genes are shaded in grey. Solid lines show direct interaction (binding/physical contact); dashed lines show indirect interaction that is supported by the literature but possibly involving ≥1 intermediate molecules that have not been investigated definitively. Molecular interactions that involved only binding are connected with a line without an arrowhead because directionality cannot be inferred.

5.5.3 Putative functions

Since gene association studies often identify surrogates for putative causal SNPs, it is imperative that data from selected SNPs be subjected to further analysis using prediction tools to short list candidates for finer analysis before causality could be established. Unless causative SNPs/genes are identified, development of targeted therapeutics are difficult to achieve. Of the 42 non synonymous polymorphisms entered into the SIFT algorithm (Table 5.19) seven SNPs had a significant score ($p<0.05$) causing an intolerant change of amino acid. Since SIFT program only evaluates non synonymous SNPs, and the majority of polymorphisms lie within the regulatory regions at 3', 5' ends and in introns, the SNPs selected using the SCAN database for insights into the potential contribution of selected SNPs on gene regulatory functions (cis-or trans effects, eQTLs) were interrogated (423-427). 132 SNPs as potential eQTLs in HapMap study populations (Caucasian, Yoruban, Han Chinese/Japanese) were identified (Table 5.20). Analysis of selected candidate SNPs for potential eQTLs revealed several target genes that are regulated both in cis- and trans, as to be expected. The fine regulation in complex biological networks by a SNP may be direct or indirect, and likely influence gene expression through short or long range interactions. The prediction for regulation of expression was limited to those showing high statistical significance ($p\text{-value}<10^{-4}$) and to any one of the HapMap populations. The promising candidates from SCAN database should be further validated by independent methods (RT-PCR) to confirm for direction and magnitude of expression changes in a tissue specific manner. In addition to pathway based candidate SNP approaches for cachexia, genome wide association studies have the potential to identify promising variants for further interrogation of the genome for genetic predisposition.

SNP	Gene	Amino acid change	Protein	Amino Acid	Using orthologues or homologues in the protein alignment
					Prediction
rs4994	ADRB3	R64W	NP_000016	R	TOLERATED
				W	POTENTIALLY DAMAGING
rs622756	HYLS1	D3Y	NP_112597	D	TOLERATED
				Y	POTENTIALLY DAMAGING
rs1800450	MBL2	D54G	NP_000233	D	POTENTIALLY DAMAGING
				G	TOLERATED
rs1800451	MBL2	E57G	NP_000233	E	POTENTIALLY DAMAGING
				G	TOLERATED
rs1801282	PPARG1	A12P	NP_056953	A	POTENTIALLY DAMAGING
				P	TOLERATED
rs2228570	VDR	T1M	NP_000367	T	POTENTIALLY DAMAGING
				M	TOLERATED
rs1800571	PPARG1	Q83P	NP_005028	Q	POTENTIALLY DAMAGING
				P	TOLERATED

Table 5.19 - Polymorphisms of potential functional significance. Of the total number of candidate SNPs (n=281) selected for an association study for the phenotype of cachexia, seven SNPs with non-synonymous amino acid substitutions were predicted by SIFT to potentially influence structure or function of the encoded proteins. SNP ID (rs#), gene name, amino acid change, protein accession number and prediction based on sequence alignment from orthologues or homologues are shown.

Table 5.20 - Potential cis- and trans effects from select candidate gene polymorphisms- potential expression changes (eQTLs) in representative genes

rs#	Chromosome	Position	Alleles	Gene	Feature [Gene Accession #]	Representative genes whose expression may be affected
rs4833095	4	38476105	C/T	TLR1	Reference[NM_003263.3]	GPR137B: MFSD7: ARHGDIA: DEF8: ZDHHC4
rs3804099	4	1.55E+08	C/T	TLR2	Coding-synonymous[NM_003264.3]	BTBD12
rs2569190	5	1.4E+08	A/G	CD14	UTR-5[NM_000591.2]	EIF4EBP1: ANKZF1:CCNB1IP1: PARS2
rs7096206	10	54201691	C/G	MBL2	Near-gene-5[NM_000242.1]	ZFAND3
rs5498	19	10256683	A/G	ICAM1	Reference[NM_000201.2]	PDSS2: UCP2: CLDN19: PCGF6: KIAA1754: NADK: FLJ45032: GLB1: PI4KB: PDK3: BST2: P2RX7: ZBTB3: ZER1: PEX19: SASH3: MTX2: ATP5SL: SNX2: C11orf49: PLTP: CYB561D2:SHMT1
rs5491	19	10246540	A/T	ICAM1	Reference[NM_000201.2]	C19orf61: P2RY10: PPP2R5D: BRF2: C12orf32: ASCC2: ENTPD2: FBXO7: FAM110A: PSMB6: RPUSD3
rs281432	19	10251658	C/G	ICAM1	Intron[NM_000201.2]	RAC2: ADO: SEMA6A: ALAD: P2RX7: SMTN: PXN: KCNIP2: LILRB4: PDE6G: RHOBTB2: MOBKL2A: LOC100130790: SLC16A3: GLTP
rs3176860	1	1.01E+08	A/G	VCAM1	Intron[NM_080682.1]	TRAPPC4

rs2227956	6	31886251	C/T	HSPA1L	Reference[NM_005527.3]	CYP1A2: HLA-DQB1: HLA-DQB2: HLA-DRB1: HLA-DRB2: HLA-DRB3: HLA-DRB4: HLA-DRB5: LOC100133484: LOC100133583: LOC100133661: LOC100133811: LOC730415: RNASE2: ZNF749: hCG_1998957: HLA-DQB1: HLA-DQB2: HLA-DRB1: HLA-DRB2: HLA-DRB3: HLA-DRB4: HLA-DRB5: HLA-DRB6: LOC100133484: LOC100133583: LOC100133661: LOC100133811: LOC730415: RNASE2: ZNF749: hCG_1998957: PTGER4
rs1130214	14	1.04E+08	G/T	AKT1	Intron[NM_001014432.1]	FUT8 YRI
rs1800625	6	32260420	C/T	AGER	Near-gene-5[NM_172197.1]	HLA-DQA1:HLA-DQA2:ATP13A2: PEX26: TUBA8: PISD: LOC100131053: PQLC2: PSCD3
rs3774932	4	1.04E+08	A/G	NFKB1	Intron[NM_003998.2]	ADRA2B: ADPRHL1
rs1801	4	1.04E+08	C/G	NFKB1	Intron[NM_003998.2]	TLE2: GDF7: WDR27: CYP4V2
rs1800629	6	31651010	A/G	TNF	Near-gene-5[NM_000594.2]	HLA-DQA1: HLA-DQA2: FAM20B: HLA-B: HLA-C: MICA: MICB: XXbac-BPG181B23.1
rs1800630	6	31650455	A/C	TNF	Near-gene-5[NM_000594.2]	GTF3C6
rs1799964	6	31650287	C/T	LTA	Near-gene-3[NM_000595.2]	GNAZ
rs909253	6	31648292	C/T	LTA	Intron[NM_000595.2]	ZNF600 : MORC2: AFTPH: HLA-DQA1: HLA-DQA2: FNDC3A: ATG2B
rs17561	2	1.13E+08	G/T	IL1A	Reference[NM_000575.3]	SLC7A5: PSAT1:LOC284702: GPX7
rs1800587	2	1.13E+08	C/T	IL1A	UTR-5[NM_000575.3]	MRPS7
rs1143627	2	1.13E+08	C/T	IL1B	Near-gene-5[NM_000576.2]	OSBP
rs2070874	5	1.32E+08	C/T	IL4	UTR-5[NM_172348.1]	MACROD1
rs2243250	5	1.32E+08	C/T	IL4	Near-gene-5[NM_172348.1]	RECQL4: LRRC45: NFKBIL2: RRAS2: C19orf47: CHTF18: EIF3H: DBF4B
rs2069835	7	22734396	C/T	IL6	Intron[NM_000600.2]	PGPEP1: PA2G4: LOC100144415: RAD9A

rs1554606	7	22735232	G/T	IL6	Intron[NM_000600.2]	C8orf59: CNR1: LNPEP: RPL23: SNORA21: LOC284702: TAS2R49: ADIPOR2: NUFIP2: EIF1AP1: EIF1AX: CUGBP1: ZNF706: BCL2L2
rs2069845	7	22736674	A/G	IL6	Intron[NM_000600.2]	C8orf59: BCL2L2: CNR1:ADIPOR2: RPL23: SNORA21: ZC3H10: LNPEP: NUFIP2: OAZ2: TGFBR1: SDCBP2: TAS2R49
rs1368439	5	1.59E+08	G/T	IL12B	UTR-3[NM_002187.2]	C3orf62: NEURL: ZNF517: SEPN1
rs2043055	11	1.12E+08	A/G	IL18	Intron[NM_001562.2]	EGFL8: PPT2: MRPL11: CEU: TWIST1
rs549908	11	1.12E+08	G/T	IL18	Reference[NM_001562.2]	KCTD11: IFNA8: SEPX1: C21orf128
rs360729	11	1.12E+08	A/T	IL18	Intron[NM_001562.2]	RPS27A: UBB YRI: UBC: SEPX1: RP9P: PVRIG: COX4I1: C19orf53: GPBAR1: IFNA8
rs3882891	11	1.12E+08	A/C	IL18	Intron[NM_001562.2]	DRAM: ZNF566: FNBP4: HERPUD1: EMG1: OTUD5: RBM19: ZNF567: SPAG7: PRDM2: C10orf2: OBFC2A: CHMP4A: MDP-1: PPTC7: CLK4: NFKBID: ZBTB43: CCDC49: ZNF460: UHRF1BP1L: BRD1: LOC90834: PI4K2A
rs2193049	12	66833189	C/G	IFNG	Near-gene-3[NM_000619.2]	NPY: TFCP2L1: C16orf33: IL1R2: IFI44L: SFRS10
rs2069727	12	66834490	A/G	IFNG	Near-gene-3[NM_000619.2]	GCLM: GALR3: RP5-1077B9.4: BTN2A3: LRRCC1: FBXL4: CASP6: ZAK: CUTA: DUSP7: MBOAT2: RAB23: PTPLB: RBPJ: ANLN: EMILIN2: TTL: NOD1: C1orf112:C6orf145:SLC4A7: CUL7:TIFA: UPK2: C6orf26: MSH5: B3GALTL: EZH2
rs2107538	17	31231893	C/T	CCL5	Near-gene-5[NM_002985.2]	HYOU1: ANG: RNASE4
rs767455	12	6321206	C/T	TNFRSF1A	Reference[NM_001065.2]	MFSD7
rs1061622	1	12175542	G/T	TNFRSF1B	Missense[NM_001066.2]	PXN YRI: GPR146: LOC100130456: LOC100134611: CA9
rs1061624	1	12189852	A/G	TNFRSF1B	UTR-3[NM_001066.2]	RAB9B

rs3397	1	12189879	C/T	TNFRSF1B	UTR-3[NM_001066.2]	LOC100130157: PIGT: RAD51AP1: TCF4: NFS1: LOC728543: TMEM116: PIP4K2B: C21orf45: OIP5: NUP37: CDKN3: TMEM169: RBM10
rs496888	1	12155393	A/G	TNFRSF1B	Intron[NM_001066.2]	SPTAN1: ALKBH3: ACLY: FLJ14154: GNG7
rs1061631	1	12191086	A/G	TNFRSF1B	UTR-3[NM_001066.2]	FCRL4: LOC100128968: MIB2: C5orf29
rs2228139	2	1.02E+08	C/G	IL1R1	Reference[NM_000877.2]	SLC37A1
rs7749390	6	1.38E+08	A/G	IFNGR1	Intron[NM_000416.2]	RHBDF2: CPSF1: LMF2: HIST1H1B: HLA-B: HLA-C: MICA: MICB: XXbac-BPG181B23.1: HIST1H1E: VIPR1: COX8A: WDR24: AMMECR1: SNORD96B: LOC100130790: SLC16A3: REEP4: RNPEPL1
rs1130864	1	1.58E+08	C/T	CRP	UTR-3[NM_000567.2]	CD84
rs3093066	1	1.58E+08	A/C	CRP	UTR-3[NM_000567.2]	MAP1B: RASSF7
rs1205	1	1.58E+08	C/T	CRP	UTR-3[NM_000567.2]	TSC2: PURA: PLAUR: ARID3C: DCTN3
rs3775485	4	74494685	A/T	ALB	Intron[NM_000477.3]	C17orf59
rs1800849	11	73397813	C/T	UCP3	Near-gene-5[NM_022803.1]	CLK3: LBXCOR1: PIAS1
rs972936	12	1.01E+08	A/G	IGF1	Intron[NM_000618.3]	TAGLN2: ARF6: CCDC85B
rs11111272	12	1.01E+08	C/G	IGF1	Intron[NM_000618.3]	C1orf86: FLJ14100: ARF6: OAZ2: KCTD5: CCDC85B
rs2453839	7	45920098	C/T	IGFBP3	Intron[NM_000598.4]	ZNF793:ZHX3
rs1801278	2	2.27E+08	A/G	IRS1	Missense[NM_005544.1]	RBL1: TIMELESS: HSBP1: ZNF558: DPYSL2
rs2234931	2	2.27E+08	A/G	IRS1	Coding-synonymous[NM_005544.1]	RBL1: DPYSL2: CHAF1B: HSBP1: ZNF558: WWP2: TIMELESS: UHRF1: ATAD5: FAM111B

rs2229765	15	97295748	A/G	IGF1R	Coding-synonymous[NM_000875.3]	WNK1: TAP1: IFI35: GNB2: ENDOG: UQCRC1: GUK1: FLJ36031: IFI6: PSMD3: IRF1: RRP9: GTF2H4: VARS2: MAP3K11: BAT5: PLAC8: HIST1H2AB: HIST1H2AE:HIST1H2BN: HIST1H3A: HIST1H3B: HIST1H3C: HIST1H3D: HIST1H3E: HIST1H3F: HIST1H3G: HIST1H3H: HIST1H3I: HIST1H3J: IDH2: GRIN1: TUBB2C: GPR172A: GMPR: POLD4: SLC9A5
rs1743966	6	1.35E+08	C/T	SGK1	Intron[NM_005627.3]	PRKCSH: SLC27A5: APLP2: OAS3: UGT2B15: UGT2B17
rs9402571	6	1.35E+08	G/T	SGK1	Near-gene-3[NM_005627.3]	CLK3: DOK7: FBXO16: ZNF395: RNF34: EFNA3: C1orf113: LOC100127947: MYLIP: NR4A1: TGM2: LOC100133227: SEPT9
rs2236903	1	2.08E+08	A/T	HSD11B1	Intron[NM_005525.2]	GIPC1: F2RL3: HERC1: FLNB: FKSG43: FRMD8
rs16940665	17	41263677	C/T	CRHR1	Coding-synonymous[NM_004382.3]	LARP5
rs17689824	17	41260178	C/T	CRHR1	Intron[NM_004382.3]	PYY
rs7209436	17	41225913	C/T	CRHR1	Intron[NM_004382.3]	TRIM46
rs110402	17	41235818	C/T	CRHR1	Intron[NM_004382.3]	TRIM46
rs780094	2	27594741	A/G	GCKR	Intron[NM_001486.2]	TNFRSF18:C16orf62: XPC: ATRN: NDFIP1: PPAPDC1B: RUVBL1: SLC25A4: TDRKH: XRRA1
rs780106	2	27535102	A/C	IFT172	Intron[NM_015662.1]	TXNDC1:PHF6: CCDC120: FGFR1OP2
rs1647266	2	27546989	C/T	IFT172	Intron[NM_015662.1]	TXNDC1: PHF6: CCDC120: FGFR1OP2

rs7310409	12	1.2E+08	A/G	HNF1A	Intron[NM_000545.4]	PDE8A: MT1H: MT1P2: LACTB2: MBP: C20orf19: OBFC1: UBE2L3: CCNY: STAU1: TULP4: HSP90B1: HSP90B2P: LRRC28: RAB3IP: RBM41: FAS: AIFM2: CAMK2G:PTER:UBE2D1: LOC731788: NF1: KPNA3: ANUBL1: SETD3: HNRNPUL1: LOC100128562: PTPN9: RFXDC2: BLMH: DDX52: DIS3L: TDRD3
rs1169300	12	1.2E+08	A/G	HNF1A	Intron[NM_000545.4]	NLRC3: TULP4: BLMH: GEN1: LOC284952: C1orf19: SRD5A1: ATG10: C20orf19
rs2464196	12	1.2E+08	C/T	HNF1A	Missense[NM_000545.4]	TULP4: NLRC3: BLMH: GEN1: LOC284952: C1orf19: ATG10: SRD5A1: CXorf10: EFCAB4B: FLJ33046: LOC100131749: TRIO: DIS3L: ELK4: HNRNPUL1: C20orf19
rs1169302	12	1.2E+08	G/T	HNF1A	Intron[NM_000545.4]	LRRFIP1: GCC2: NLRC3: HIST1H2BC: HIST1H2BE: HIST1H2BF: HIST1H2BG: HIST1H2BI: CALML4: C11orf47: CCDC35: DNHD1: C14orf65: LOC100128667
rs1815739	11	66084671	C/T	ACTN3	nonsense[NM_001104.1]	TPPP
rs2239179	12	46544033	A/G	VDR	Intron[NM_001017535.1]	HDAC9
rs731236	12	46525024	C/T	VDR	Coding-synonymous[NM_000376.2]	SLC27A1: NUDT9: ZNF524: WIPF1: UBE2K: PRPS2: OTUD6B: PRPF38B
rs4343	17	58919763	A/G	ACE	Coding-synonymous[NM_152830.1]	DENND2D: APOM: B3GALT4: CDC37L1
rs4329	17	58917190	A/G	ACE	Intron[NM_152830.1]	FXYD2: BLK: CLU: SHROOM3: HEY1
rs2854464	12	50675158	A/G	ACVR1B	UTR-3[NM_004302.3]	NNT: AFAP1L2: ELK1
rs16892496	8	1.1E+08	A/C	TRHR	Intron[NM_003301.2]	CAT: BAIAP3
rs2070802	9	34546453	A/T	CNTFR	Intron[NM_001842.3]	EOMES: OASL: IFITM1: BIN2: SHKBP1: PRIC285: MYO18A: TIAF1: LOC399491: NPIP: PKD1

rs361072	3	1.4E+08	A/G	PIK3CB	Near-gene-5[NM_006219.1]	CCDC94: GTF2F1: LOC100130856: MAP3K14
rs2536	1	11089300	C/T	FRAP1	UTR-3[NM_004958.2]	PGK1: RAD23A: ASB7: LOC145758: PARVB: FRAT2: KIAA0182
rs1190584	14	1.02E+08	C/T	WDR20	Intron[NM_181308.1] Intron[NM_181302.1] Intron[NM_144574.2]	HBM
rs744166	17	37767727	C/T	STAT3	Intron[NM_213662.1]	SAMD10: LOC51149: TLN1: SMO: NDST1: MMP11: ABHD6: RAPGEF5
rs8069645	17	37748428	A/G	STAT3	Intron[NM_213662.1]	COMMD5
rs2293152	17	37735055	C/G	STAT3	Intron[NM_213662.1]	HLA-DQA1: HLA-DQA2
rs1053005	17	37719436	A/G	STAT3	UTR-3[NM_213662.1]	C3orf59: ENC1: BTG2
rs2419621	10	1.14E+08	C/T	ACSL5	Intron[NM_203379.1]	COCH
rs2701896	13	40025892	C/G	FOXO1	Near-gene-3[NM_002015.3]	LAMP1: CCND1: GLTSCR2: SNORD23: ADA: PIK3R5: ERAL1: C19orf22: SGTA: PES1: DCTN2: MRPL45: MRPL40: VAV1: B3GNTL1: TOMM34: PPARD: SIRPA: HPS1: PRMT6: HCLS: MRPS7: FBXO46: TH1L: GRAMD1A: GRAMD1B: NADSYN1: C11orf24: AP3D1: FOXK2: UBTD2: C1orf142: JMJD4: CDH1: CLYBL: PHC2
rs2297627	13	40131931	C/T	FOXO1	Intron[NM_002015.3]	LRRK1: CD96: LRRK1: PKN3: TLR4: MGC39372
rs2802292	6	1.09E+08	G/T	FOXO3	Intron[NM_001455.3]	RPL27A: hCG_21078: UHRF2
rs3791783	2	1.91E+08	C/T	MSTN	Intron[NM_005259.2]	C3orf59
rs2268757	3	38480857	C/T	ACVR2B	Intron[NM_001106.3]	FKBP2: ZAR1

rs947712	1	2.17E+08	A/G	TGFB2	Intron[NM_003238.1]	PLP2: RAB11FIP2: ERGIC1: LHPP: LOC100132892: DNASE2: DTWD2: LOC440983: WWTR1: STK40: WBP2: LAPTM4A: GTPBP1: TMEM59: ENPP7: LRRC33: EFHA1: CUTA: NLRP1: CAPN2: MCM9: SAMD14: MED28: RAB7A: TSG101: TESK2: KIAA0141: H1FX: C16orf44: IFITM1: ANXA5: JAM2: GNPDA1: TMED4: ASB8: TMUB1: TEX264: NUDT12: C20orf19: ING3: ADCK2: GMPR2: IQGAP2: DYNLRB1: HBP1: SRD5A3: CENTA1: C11orf49: EMP3: PIAS3: MTX2: MRPS22: NPC2: TMEM90A: NIPSNAP3A: NR1D2: NAIF1: PXN: CAPZB: LOC100130193: LOC644075: GTPBP10: PPAP2C: RNPEPL1: IMPACT
rs1890995	1	2.17E+08	C/T	TGFB2	Intron[NM_003238.1]	NUDT4: NUDT4P1: C15orf24: LOC201164: ARID3C: DCTN3: TOX4: ARMCX3: CCDC12: PWP1
rs817529	2	33350839	A/G	LTBP1	Intron[NM_000627.2]	SDF4: FICD: LRRC59: RBM15: DHX30: E4F1: PLEKHB2
rs2577262	2	11856397	C/T	LPIN1	Intron[NM_145693.1]	CD320:LRDD
rs1801253	10	1.16E+08	C/G	ADRB1	Missense[NM_000684.2]	CREBBP: RICH2: ZNF318: MED14: CBX5: MAP3K15: SH3KBP1: KIAA1267: LOC100132495: NOTCH2: NOTCH2NL: SKAP1: TXNDC13: TNFSF12: TNFSF12-TNFSF13: TNFSF13: RAD23B: TAOK3: LOC643475: PIP4K2A: PROK2: CD24: COL6A1
rs1042713	5	1.48E+08	A/G	ADRB2	Missense[NM_000024.4]	LMF1 CEU 5e-05
rs1042714	5	1.48E+08	C/G	ADRB2	Missense[NM_000024.4]	ARPC1B: FHOD1: MYD88: PODXL2: RARG: CORO1A: LOC606724
rs1042717	5	1.48E+08	A/G	ADRB2	Coding-synonymous[NM_000024.4]	LAPTM4B: DSCR6

rs1042719	5	1.48E+08	C/G	ADRB2	Coding-synonymous[NM_000024.4]	DSCR6
rs5443	12	6825136	C/T	GNB3	Reference[NM_002075.2]	SRRM2: C4orf8: RNF4: KIAA0182: ND5: UNQ6228
rs1800590	8	19840951	G/T	LPL	UTR-5[NM_000237.2]	FLJ10232: LY9: FAM102A
rs326	8	19863719	A/G	LPL	Intron[NM_000237.2]	PAPD5: ARF4
rs157580	19	50087106	A/G	TOMM40	Intron[NM_006114.1]	NUDT9: SS18L2: C11orf46
rs1862513	19	7639793	C/G	RETN	Near-gene-5[NM_020415.2]	CYFIP2: LRP8: CSF3: ZDHHC18
rs1501299	3	1.88E+08	A/C	ADIPOQ	Intron[NM_004797.2]	TBXAS1: SESTD1: WWP2: PAG1: B3GNT7
rs16928751	12	1760460	A/G	ADIPOR2	Coding-synonymous[NM_024551.2]	EMR2: CTSW
rs1137101	1	65831101	A/G	LEPR	Reference[NM_002303.3]	FAM128A: FAM128B: LOC646836
rs1137100	1	65809029	A/G	LEPR	Reference[NM_002303.3]	C16orf74: CKB: TRAF2: PTPRS: MAN1B1: MAP2K6
rs8179183	1	65848540	C/G	LEPR	Missense[NM_002303.3]	RAB11FIP3: ZNF416: TRAF7: IRF2BP1: HPS6: PI4K2A: INPP5E: TOB1: GSTM1: GSTM2: GSTM4: TM9SF4
rs12409877	1	65716460	A/G	LEPR	Intron[NM_002303.3]	GSTM1: GSTM2: GSTM4: APOA2
rs696217	3	10306457	G/T	GHRL	Missense[NM_016362.2]	ASB3: LOC728653: MECP2: RRAS2: ACBD3: OBFC2A: SRPRB
rs35681	3	10304377	A/G	GHRL	Intron[NM_016362.2]	PTGIR
rs3746619	20	54257212	A/C	MC3R	Missense[NM_019888.2]	POU5F1: POU5F1P1: POU5F1P3: POU5F1P4: SNRP70: KLF9: SPIB
rs3827103	20	54257436	A/G	MC3R	Missense[NM_019888.2]	SNRP70: POU5F1: POU5F1P1: POU5F1P3: POU5F1P4: SPIB: KLF9
rs1875233	16	55244205	A/G	MT1B	Intron[NM_005947.2]	ZC3H3: ZNF696: NAPRT1: TAF5: CRLF3: NFYB: TM9SF3: HTRA1: GMCL1: GMCL1L: KRI1: TMEM156: AZU1: SEDLP: TRAPPC2: ZNF547

rs10636	16	55200844	C/G	MT2A	UTR-3[NM_005953.2]	HIP1: KCNK1
rs3745601	19	10085548	A/G	P2RY11	Missense[NM_002566.4]	ARSA: PNPLA2
rs10183022	2	2.37E+08	A/G	CXCR7	Intron[NM_020311.2]	SSR2: IK: ATP6V0E1: SNORA74B: PFDN6: EIF4E2: VAMP8
rs1045879	2	2.37E+08	C/T	CXCR7	Reference[NM_020311.2] Coding-synonymous[XM_001714208.1]	IK: H3F3A: H3F3B: LOC440093: MRPL27: EEF1A1: EEF1AL3: EEF1AL7: CSAD: PARD6B: HNRNPH1: FOXK2: VASP: HNRNPH3: GPR44: EIF4E2: TARDBP
rs6805421	3	42703148	A/G	KBTBD5	Reference[NM_152393.2]	CMBL
rs123509	3	42708472	A/G	KBTBD5	Missense[NM_152393.2]	RIMS4
rs4855881	3	49690450	C/T	APEH	Intron[NM_001640.3]	FLJ34077
rs2142234	6	31747108	C/T	LY6G5B	Intron[NM_021221.2]	FCN1: TMEM170: ERH
rs1266076	6	31748497	A/C	LY6G5B	Near-gene-3[NM_021221.2]	B3GNT7:CMPK2: PRIC285: TTYH3: CRIM1: IFIT3: ALDH2: HOXB3: CRIP3: CCR6: CD72: OAS3: C1orf161: ZNF318: OASL: A4GALT: BLVRB: NME4: SLC45A3: SDC4: HOOK1: LOC399491: NPIP: PKD1: RTKN: EPHB1: SYS1: SYS1-DBNDD2: SPIB: PLCB2: GLIS2: DDX60: CHDH: HNRPLL: BIN1: DBN1: PLA1A: GMPR: LCK: MOBKL2B: HNF1B: CACNB3: SOX4: ISG15: DUSP4: MARCKSL1: LOC100128950: PIGZ: PARD3: LOC100134130: LOC440456: PLEKHM1: KIAA0746: SERINC2: GSTP1: ETV4: CHL1: PHLDA1: SLCO4C1: APLP2: GPER: C20orf59: CLCF1: MAP1B: CHST11: PARP12
rs3802427	9	34608641	A/G	DCTN3	Intron[NM_024348.2]	GARNL4:C10orf10
rs3088241	11	1.25E+08	C/G	HYLS1	Intron[NM_145014.1]	NCOA3
rs3848017	13	97828590	C/T	FARP1	Intron[NM_005766.2]	C9orf148: TTLL11
rs584800	13	97836088	A/G	FARP1	Intron[NM_005766.2]	SLAMF1: ABCC4
rs4969484	17	77608150	G/T	GPS1	Intron[NM_212492.1]	C20orf149

rs3826785	19	10088149	C/T	EIF3G	Intron[NM_003755.3]	ARSA: PNPLA2: BNIP3L
rs11085824	19	12862547	A/G	GCDH	Near-gene-5[NM_013976.2]	DHRS1: TADA3L: RICH2: SF3B4: C1GALT1C1: TOR3A
rs9384	19	12871643	G/T	SYCE2	Intron[NM_001105578.1]	TADA3L: SF3B4
rs163781	20	56997160	A/G	TH1L	Intron[NM_198976.1]	TNNT2: FBXL8: C6orf192: MSH3: GPR27: ZBED4: GJA4: ZNF131: ZNF184: ZNF204: NFXL1: MTMR15: C4orf30: C10orf28: MANBAL: CDC42BPG: RARRES3:KCTD20: C4orf1405:CHD1L
rs2284890	3	1.97E+08	A/G	TFRC	Intron[NM_003234.1]	MGMT
rs3817672	3	1.97E+08	A/G	TFRC	Missense[NM_003234.1]	MGMT

5.6 Discussion

Even with the same tumour type and burden, one individual may become cachectic whereas another will not. One mechanism for such variation may relate to host genotype. Genetic variation in immunity and associated signaling pathways is known to relate to outcomes in major sepsis (742), and recent findings suggest a similar pattern in cancer cachexia (207). Single-nucleotide polymorphisms in the IL-1, IL-6, and IL-10 genes that are linked to production rates of these cytokines have been associated with the prevalence of cachexia in gastric or pancreatic cancer (743). For example, the 1082G allele in the IL-10 promoter has been validated as a procachectic genotype in an independent cohort (200, 208). IL-10 has been shown to be elevated in a Myc/mTOR-driven murine model of cancer cachexia (744), as well as in cachectic patients with colorectal cancer (745). Others have identified associations with cachexia defined as >10% weight loss and polymorphisms in cytokine genes such as the IL1-B 3954C/T polymorphism (rs1143634) in patients with gastric cancer (209). Cancer related anorexia has been associated with the TNF-308G/A polymorphism (rs1800629) in patients with non-small cell lung cancer (198). Most recently, the C allele of the rs6136 polymorphism in the P-selectin gene has recently been associated with weight loss in a large heterogenous group of cancer patients and validated in an independent cohort (207). Taken together, these findings are consistent with a key role for the immune system in the variable presentation of cachexia. However, cancer cachexia has been defined in a number of ways (6-10), mainly focused on weight loss and the presence of systemic inflammation. This has lead to phenotyping in many of the existing studies on genetic association relying wholey on a degree of weight loss and inflammation. However, one recent definition described cancer cachexia with a more musculocentric view (11). This definintion highlights the importance of skeletal muscle loss as one of the most significant event in cachexia and is associated with a poor outcome (11, 12). In genetic studies of common diseases, the capacity to define genotypes is often far better than the capacity to define phenotypes, therefore more robust classifications of phenotypes need to be sought. With the existing studies focusing mainly on weight loss and CRP to define cancer cachexia phenotypes this may have lead to the discovery of associated SNPs exclusively involved in mediating the immune response. Recent advances in the use of routine CT scans in cancer patient's care have lead to unmasking of detailed body composition analysis for accurate measurements of skeletal muscle and adipose tissue mass (746). Taking

into account a level of skeletal muscle mass alongside degrees of weight loss and inflammation will improve accuracy of phenotyping and may open up the analysis to include genetic variants in muscle specific genes.

The published studies linking genetic variation to cancer cachexia are listed in Table 5.1, the definitions of the cancer cachexia phenotype vary greatly from weight loss cut offs and inclusion of systemic inflammation, to performance status assessment and quality of life scores. The only study to directly state and include an independent validation cohort to confirm the presence of a significant SNP is the detection of the p-selectin genotype from our group (207). In the discovery cohort a further 20 significant SNPs were found however these did not reach significance in the validation cohort. It may be these SNPs are significant but due to the size of the independent validation cohort they failed to reach significance.

In the current review, associated polymorphisms in genes with a possible role in cachexia have been recorded as well as polymorphisms with clinical associations related to cachexia such as inflammation, weight/body composition changes and cancer survival. Because cancer cachexia is a multifactorial disease involving a variety of biological pathways it can be assumed that analysis of combinations of gene variants encoding interacting factors within a biological chain or cascade, rather than isolated investigation of its single components, may have more chances to reveal real causative connections between gene polymorphisms and phenotypes. Of the 80 polymorphisms with a potential role in the development of cachexia that have been independently verified in at least one repeat study, 24 polymorphisms have been shown to have more than one association on clinical features associated with cancer cachexia. These 24 polymorphisms are likely to be the most promising candidates in terms of susceptibility biomarkers of cancer cachexia and have investigated in Chapter 6.

Eighty eight newly identified genes with a potential role in cancer cachexia were included since the last review, however the main limitation to identifying new SNPs in these genes was the lack of studies to date into functional polymorphisms within them. Undertaking a genome wide association study (GWAS) would be one way to overcome this potential limitation and in the future would be preferable to repeating candidate gene selection studies. However, without an adequate sample size and highly accurate phenotyping, coupled with lack of government funding to complete

the project, candidate gene association studies will continue to provide novel insights in to the genetic of cancer cachexia.

In conclusion, the current review has expanded on an initial framework to further enhance the possibility of identifying functional polymorphisms involved in cancer cachexia. Based on the expansion of the definition of cancer cachexia along with an inclusion of skeletal muscle mass and not just weight loss and systemic inflammation, new muscle specific SNPs may provide novel biomarkers in the early detection of individuals at risk of developing cancer cachexia. These SNPs are explored in an extensive association study described in Chapter 6.

CHAPTER 6

Genetic signatures associated with susceptibility to cancer cachexia vary according to weight-loss or low muscle mass phenotype

6.1 Summary

6.1.1 Aims

Cancer cachexia is characterised by loss of weight (WL), muscle and fat. This Chapter aimed to (i) replicate SELP rs6136 and other genes previously associated with WL (ii) explore associations with 92 predefined new candidate single nucleotide polymorphisms (SNPs) and (iii) explore a panel of candidate SNPs (i and ii) for association with CT-defined low muscularity (LM) +/- WL. Whether the transcription in muscle (n=134 cancer patients) of identified genes was altered according to cachexia phenotype was also explored.

6.1.2 Methods

The replication study included 545 new cases. Combined analysis of prior and new cohorts (n=1276) explored associations of new candidate SNPs with WL and LM. Human muscle transcriptome was analysed using Agilent platform.

6.1.3 Results

SNPs rs6136 (SELP) and rs4149570 (TNFRSF1A) shown in a prior study to associate with WL, were replicated. New candidate SNPs in the following genes showed association with WL: IFT172, ACVR2B, TLR4, FOXO3, IGF1, LEPR, FOXO1, TOMM40, and CPN1. SNPs in ACE, MT2A, WDR20, PPARG, LPIN2 and LEPR, ACVR2B, TNF, ACE were associated with LM and concurrent LM + WL, respectively. There was concordance between muscle-specific expression for ACVR2B, FOXO1 and 3, LEPR, PPARG, TLR4, TNFRSF1A and TOMM40 genes and LM or WL ($P<0.05$).

6.1.4 Conclusions

rs6136 in the SELP gene is the leading replicated SNP to associate with WL. New SNP associations for cachexia phenotypes that include an index of muscle mass and

muscle specific gene expression signatures for WL or LM provide insights into potential risk factors/biomarkers for muscle loss in cancer cachexia.

6.2 Introduction

As previously stated, cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life and duration of survival (747). Cachexia is a complex multifactorial syndrome characterized by weight loss (WL), and specific losses of muscle and/or adipose tissue (748). Based on current knowledge of demographic and clinical factors including cancer type, it is not possible to predict, for any given cohort of patients, who will develop cancer cachexia and who will not. Such variation may, in part, be due to the patient's genotype. Knowledge of genotypic variation associated with cachexia could contribute to early identification of patients at risk and allow institution of prophylactic measures.

Using a candidate gene approach, prior research has identified an association with cancer cachexia and several single nucleotide polymorphisms (SNPs) on different genes/chromosomes; among these, a variant from the SELP gene (that encodes for the cell adhesion molecule P-selectin (207)) was investigated in depth for functional significance. Since that study, many new target genes that might potentially influence the development of cancer cachexia have been reported (89, 103, 145, 283, 286, 329, 749). Chapter 5 details a comprehensive and contemporary review of candidate genes and polymorphisms to aid association studies for the identification and characterisation of genetic determinants of the different phenotypic domains described in cachexia (193, 750). Candidate genes and their polymorphisms considered in the present study are from pro/anti-inflammatory pathways, neuronal melanocortin signalling pathways, energy regulation, appetite regulation, muscle, and adipose tissue catabolic pathways.

Although there is depletion of both adipose tissue and lean body mass in cancer cachexia, WL per se has long been used as the diagnostic criterion for the syndrome (751) and this remains the case in current classification systems (11). Validity for the use of WL in the diagnostic phenotype is provided by the observation that it is an

independent predictor of shortened survival, with increasing WL carrying a progressively worse prognosis (23, 396).

However, it could be argued that it is skeletal muscle loss that has the greatest impact on patients' function and quality of life. It has recently been possible to specifically quantify muscle mass in cancer patients' diagnostic CT scans and low muscularity (LM) identified by such means is clearly associated with a poor outcome in advanced cancer (11, 12, 69). One limitation of this approach is that due to the absence of pre-illness scans, it is not possible to document a state of active muscle loss but rather one of LM determined by pre-determined cut-offs. In the present study the term LM is used in a manner synonymous with that of sarcopenia defined by cut-offs in relation to excess mortality (28, 396). The combination of LM and WL has been suggested to combine a focus on muscle mass with a dynamic process of active loss (11). Interestingly, such a combined definition proved superior to its individual components in terms of identification of cancer patients with histological evidence of skeletal muscle fibre atrophy (Chapter 4) (752). Therefore both WL, LM and a combination of the two were used in the characterisation of the cachexia phenotype.

A candidate gene approach was used to evaluate the association between genetic polymorphisms and the presence of cancer cachexia in patients from Europe and Canada. The hypothesis was that inter-individual variations in the susceptibility to cachexia are in part due to inherited genetic variations (host); remaining phenotypic variance may be ascribed to the contribution of the tumour or other comorbidity. The premise that susceptibility variants may show a certain degree of overlap with related sub-phenotypes was also tested (e.g., WL cut-offs adopted to describe severity of cachexia). The latter is an expected consequence for a polygenic trait such as cachexia and the underlying cross-talk from the biological networks involved (18). The primary objective of the present study was to further replicate findings from a previous association study which identified the SNP rs6136 in the SELP gene and a number of other polymorphisms as promising candidates influencing genetic predisposition to cachexia. The second objective was to explore the association with 92 predefined new candidate SNPs and WL. The third objective was to compare our entire panel of candidate SNPs and their association with LM with and without WL. Lastly, it was investigated whether genes demonstrating significant associations with the cachexia phenotypes had altered transcript expression in muscle from cancer patients with or without those phenotypes.

6.3 Patients and Methods

(Detailed methodology is found in Chapter 3)

6.3.1 Genotyped cancer patients: new and prior study cohorts

Study subjects were recruited between 2004 to 2012 from the National Health Service Lothian, UK; Cross Cancer Institute, Edmonton, Canada; McGill University Health Centre, Montreal, Canada; Palliative Research Centre, Norwegian University of Science and Technology (NTNU), Norway; Cantonal Hospital, St Gallen, Switzerland; and Department of Medical Oncology, University Hospital of Larissa, Greece. All subjects participated in clinical or research studies on cancer cachexia at the host institutions under ethically approved protocols allowing for analysis of patients' DNA. Recruitment was conducted at first presentation (treatment naive) to surgical, oncology or palliative care clinics at each institution. Recruitment was performed sequentially with the following exclusion criteria: (i) under 18 years of age; (ii) cognitive impairment; (iii) inability to give written, informed consent; (iv) presence of underlying infection; (v) on corticosteroids. Patients recruited generally had cancer types with propensity to develop cachexia (e.g. gastric/oesophageal, pancreatic, lung). Overall, 1276 patients were included: 731 from our prior study and 545 new patients. More than 98% of the patients were of European descent. Information collected on each patient included date of birth, date of diagnosis, type and stage of cancer. All patients underwent measurements of height and weight at the time of recruitment. Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. WL was calculated and expressed as percentage of pre-morbid body weight lost. The documentation of WL (current minus previous recalled stable weight) is dependent on the accurate recall of previous weight. Studies in healthy populations suggest a strong correlation between current recalled and measured weight (753). Height and weight were used to compute BMI (kg/m^2); this was calculated for time of recruitment (at time of diagnosis of cancer). Diagnostic or staging CT scans closest to the time of diagnosis (within 30 days on average) were selected to determine the level of muscularity. Stage of disease was based on the American Joint Committee on Cancer stage groupings I, II, III and IV. All patients provided written informed consent to allow analysis of their DNA.

6.3.2 Skeletal muscle transcriptome study

Description of the patients who contributed to the muscle transcriptomic biobank has been described recently (754). Briefly, *rectus abdominis* muscle (0.5 – 1 g) from adult cancer patients were obtained during open abdominal surgery. Review of medical charts and CT images was used to identify WL status and muscularity.

6.3.4 Phenotypes

- WL >5%, >10%, >15%. A range of weight loss was used to provide a subgroup analysis that would help identify associations that would have been missed with a single cut-off as the interest is to detect all potential associations in a polygenic model of inheritance where the variants are likely to be of lower penetrance, yet conferring finite effects.
- LM: analysis of CT scans allows patients to be classified as having LM or not. Cut offs for LM were defined in relation to the duration of survival of advanced cancer patients (396).
- LM with any degree of WL (>2%)

6.3.5 CT Analysis

Digitally stored CT images completed with a spiral CT were analysed as described previously. A transverse CT image from the third lumbar vertebrae (L3) was assessed for each scan date and tissue cross-sectional area (cm^2) quantified (395). All CT images were analysed by a single trained observer. Cross-sectional area for muscle was normalized for stature (cm^2/m^2) and a lumbar skeletal muscle index (SMI) computed as described previously (28, 397). SMI cut offs for LM were based on a CT-based study of cancer patients by Martin et al. (396).

6.3.6 Candidate gene and SNP selection

All candidate genes and SNP selections were based on the systematic literature review presented in Chapter 5 (193, 750). Candidate SNPs met one or more of the

following criteria: previously published association with cancer cachexia (197, 200, 209), statistically significant association with cancer cachexia in our prior study but still requiring validation (207), likely role in cancer cachexia based on functional or clinical relevance in more than one study (193, 750), significant SNPs identified in a preliminary study (755), and finally those SNPs which had been identified in relation to pro/anti-inflammatory pathways, neuronal melanocortin signalling pathways, energy regulation, appetite regulation, muscle, and adipose tissue catabolic pathways since the prior systematic review (193, 750).

6.3.7 Genotyping

Genotyping of the samples were performed on the Sequenom iPLEX Gold platform (San Diego, CA, USA) or TaqMan assay (for rs4280262) using services from the McGill University and Genome Quebec Innovation Center, Montreal, Quebec, Canada. Polymorphisms selected were validated for assay feasibility using DNA from apparently healthy Caucasian subjects (n=92) from Coriell Institute (Coriell Panel, Coriell Institute of Medicine, CA, USA) (398).

6.3.8 Microarray analysis

Microarray analysis was conducted as previously described(754). Briefly, total RNA was isolated, purified and subsequently quantified. RNA samples with RNA Integrity Numbers (RIN) greater than 7.0 were used. RNA was subjected to linear amplification and Cy3 labeling and Hybridization to Agilent Whole Human Genome Arrays using Agilent kits (One Color Low RNA Input Linear Amplification Kit Plus, One Color RNA Spike-In Kit and Gene Expression Hybridization Kit) according to the manufacturer's protocols. The arrays were scanned using an Agilent Scanner, the data was extracted and quality was evaluated using Feature Extraction Software 10.5.1 (Agilent). The data were normalized using GeneSpring GX 11.5.1 (Agilent). The data used in this publication have been deposited in the U.S. National Center for Biotechnology Information (NCBI) Gene Expression Omnibus25 and are accessible through GEO series accession number GSE41726.

6.3.9 Power calculations

Power calculations were performed using Quanto. For the most prevalent cachexia phenotype (i.e. >5% WL, 50% affected), the present study has 87% power to detect an OR of 1.5 for SNPs with a MAF of >0.05. For the least prevalent cachexia phenotype (i.e. >15% WL, 16% affected), the present study has 35% power to detect an OR of 1.5 for SNPs with a MAF of >0.05.

6.3.10 Statistical analysis

6.3.10.1 Gene association study

Statistical analyses were performed using PLINK (version 1.06) (399). Unconditional logistic regression was employed to calculate ORs and their 95% CI for the minor allele of individual SNPs and its association with each proposed cachexia phenotype. All analyses were adjusted for covariates that may affect WL: age at diagnosis, sex, pre-diagnosis BMI, tumour type and stage.

Patients meeting the criteria for each of the proposed cachexia phenotypes were compared with patients who had lost <5% body weight as control.

To account for multiple testing, permutation testing was performed by running the adaptive permutation test in PLINK within each proposed phenotype. Permutation tests are often employed to adjust groups of correlated tests for multiple testing, since conventional methods such as Bonferroni correction are overly conservative when tests are correlated (400). The adaptive permutation test in PLINK gives up permuting SNPs that are clearly not going to be significant. This greatly speeds up the permutation procedure, as SNPs that are not significant will drop out quickly, making it possible to evaluate properly the significance of the handful of SNPs that require millions of permutations.

Finally, candidate genes (and the SNPs in the corresponding gene regions) were grouped based on known functional similarity according to gene ontology using AmiGO (Table 6.1). The set-based test in PLINK was used to analyse association

between grouped SNPs and cachexia = phenotypes. The set-based test selects the best set of SNPs whose mean of these single SNP statistics is significant after permutation, which is particularly suited to large scale candidate gene studies (401). The empirical p-values of the set-based test were obtained by a permutation of 10 000 times of phenotype labels.

Table 6.1 - Candidate genes groupings based on known functional similarity according to gene ontology

Regulation of MAPK activity		
GHRL	IL1B	TNF
PIK3CB	IGF1	SGK1
SELP	TLR4	IGF1R
FOXO1	TRAF6	IRS1
FOXO3		
Regulation of apoptosis		
TOMM40	IGF1	PPARG
VDR	CNR1	IGF1R
TLR2	CNTFR	IKKA
TNF	DIO1	FOXO3
IL10	TNFRSF1A	IL1B
IL6R	TRAF6	VCAM1
Regulation of NF-KB activity		
IL1B	TNF	TLR4
IL10	TLR2	TNFRSF1A
IKKA	IL6R	TRAF6
Inflammatory response		
GHRL	CRP	TNF
TNFRSF1A	TRAF6	IL10
IL1B	AGER	TOMM40
CNR1	FOXO3	IL6R
LRRFIP1	PPARG	RUNX1
SELP	STAT3	TLR1
TLR2	TLR4	TNFRSF1B
Lipid metabolism		
PPARG	CRP	TNF
ADIPOR2	LPL	IRS1
IL1B	TOMM40	CNR1
FTO	LEPR	LIPE
LPIN2	TRAF6	VDR

Appetite regulation		
GHRL	LEPR	CNR1
MC3R	MC4R	NPY
STAT3	VDR	
Cell adhesion		
IL18	ICAM1	VCAM1
TNF	SELP	TNF
AKT2	AZGP1	IL10
NPY	PIK3CB	STAT3
Protein metabolism		
TNF	ACE	TRAF6
TSC2	VCAM1	VDR
IGF1	IL10	RUNX1
PIK3CB	PPARG	ACSL5
APEH	IL1B	CPN1
MC4R	CNTFR	TNFRSF1A
TNFRSF1B	MSTN	IGF1R
IL6R	GHRL	
Immune response		
IL10	TNF	RUNX1
SELP	STAT3	TLR1
TLR2	TLR4	PPARG
TNFRSF1A	TRAF6	VCAM1
TOMM40	FOXO1	FOXO3
AZGP1	CNTFR	CRP
ICAM1	IGF1	IGF1R
IKKA	IL1B	IL6R
IRS1	LPL	LRRFIP1
MT2A	PIK3CB	IL18
ATP Binding		
AKT2	CAMK2B	IGF1R
PIK3CB	RUNX1	STAT3
Calcium signalling		
STAT3	PIK3CB	RUNX1
TOMM40	CRP	GHRL
LTBP1	NPY	VDR
TNF		
Cell membrane structure and function		
ACE	ADIPOR2	AGER

APEH	TOMM40	AZGP1
CNR1	CNTFR	CRHR1
FOXO3	HYLS1	ICAM1
IGF1R	IL6R	IRS1
LEPR	LPL	LRRFIP1
MC3R	MC4R	PIK3CB
SELP	STAT3	TLR1
TLR2	TLR4	TNF
TNFRSF1A	TNFRSF1B	TRAF6
TRHR	VCAM1	
Signal Transduction		
CAMK2B	IL1B	IRS1
NR3C1	MSTN	MT2A
NPY	PIK3CB	RUNX1
SELP	IGF1	TLR1
TLR2	TLR4	SGK1
STAT3	TNF	TNFRSF1A
TNFRSF1B	TRAF6	TRHR
TSC2	VCAM1	VDR
AGER	PPARG	LEPR
ACSL5	ADIPOR2	AKT2
TOMM40	AZGP1	CNTFR
CRHR1	CRP	FOXO1
FOXO3	FTO	GHRL
HNF1A	ICAM1	IGF1R
IKKA	IL10	IL6R
INHBC	LIPE	LRRFIP1
LTBP1	MC3R	MC4R
Metal ion regulation		
ACE	CPN1	CRP
FTO	MT2A	NR3C1
PPARG	VDR	
Insulin signalling		
GHRL	IRS1	TNF
IGF1	TSC2	STAT3
TNF	PPARG	IL1B
AKT2	TOMM40	AZGP1
CNR1	FOXO3	IFT172
IGF1R	HNF1A	PIK3CB
Transcription Regulation		
AZGP1	FOXO3	HNF1A

IL10	IL1B	LPIN2
LRRFIP1	MSTN	NR3C1
PPARG	RUNX1	STAT3
TLR2	TLR4	TNF
TNFRSF1A	TRAF6	VDR
Glucocorticoid signalling		
CPN1	IL10	TNF
GHRL		

6.3.10.2 Transcriptomic study

Pearson correlation analysis was conducted to assess the linear relationship between the considered phenotypes independently (SMI or WL) with the expression of transcripts from select candidate genes. T-test analysis was conducted to compare how SMI (or WL) values differed for patients with high expression versus those with low expression for each of the candidate genes. The high and low expression groups for each gene transcript were determined by sorting patients by expression intensity and subsequently splitting the patients into three equally sized groups: those with high expression, mid- expression and low expression. The extremes of gene expression were compared while leaving out middle values. Comparison of the distal ends of gene expression maximizes the chance of detecting differences. Given the inherent variation in measures of gene expression, separation of the compared categories in this fashion also limits potential classification error. The cases considered for SMI and WL phenotypes for gene expression were based on sorting of transcript expression in all of the samples and binning based on extremes of gene expression as described above. The samples used for SNP studies (based on WL or muscularity) are non-matched as these two were independently designed studies. Transcriptome profiles were obtained from a single biopsy specimen along the disease trajectory of patients (static but not a dynamic read-out). Caution was exercised in over-interpreting the data with the emphasis to seek nominal relationships as it was not possible to delineate the expression from early vs. late response genes. A mechanistic understanding of the role of early and late response genes and their spatiotemporal relationships could be clearly discerned in animal models of cachexia(207), as described earlier.

6.4 Results

6.4.1 SNPlexing

Of the 148 SNPs selected initially (21 SNPs from a previous association study and 127 newly selected SNPs for this study), for Sequenom platform, 15 SNPs failed at the multiplex assay design stage, and 15 SNPs were non polymorphic, leaving 118 SNPs for genotyping in the present study population. Assay duplicates for 154 samples were included and genotyped for all 118 SNPs; 100% concordance of

genotype calls for replicates were obtained. Of the 1452 patients samples available for the study, detailed clinical annotations for the study end points were available for only 1276 patients. Therefore, germline DNA isolated from buffy coat cells from these 1276 individuals were interrogated for the 118 SNPs. SNP call rates in the study population with >90% were retained for all subsequent analysis (2 SNPs did not meet this criteria; rs4280262 and rs1544410 and these showed call rates of 80 and 86% respectively). Three SNPs showed a minor allele frequency of <5% and these were excluded (rs1805086; rs2536; and rs16139) leaving 113 SNPs from a total of 62 genes (Table 6.2) amenable for association analysis. Deviations from Hardy-Weinberg equilibrium (HWE) were assessed in the Coriell panel of control population using the χ^2 test with 1 degree of freedom (df); a p-value of <0.001 was considered as a significant deviation from the HWE proportions. None of the 118 SNPs considered for association analysis showed deviations from HWE in the control Coriell samples or in the study subjects.

Table 6.2 - List of polymorphisms considered for association analysis								
SNP ID, rs#	CR	MA	Strand sequenced	MAF	HWE P in cases	Gene	Position of SNP	Source of SNP
rs1025333	**0.98**	**A**	**F**	**0.08**	**1.04E-02**	**IRS1**	**Intron**	**Previous association**
rs10441113	**0.99**	**T**	**R**	**0.37**	**7.96E-01**	**CAMK2B**	**Intron**	**Previous association**
rs1049353	**1.00**	**A**	**F**	**0.26**	**9.65E-01**	**CNR1**	**UK**	**Previous association**
rs1053005	1.00	C	R	0.19	6.67E-02	STAT3	UTR-3	Current study
rs10636	**0.99**	**C**	**F**	**0.26**	**5.47E-01**	**MT2A**	**UTR-3**	**Preliminary study**
rs110402	0.99	A	R	0.45	2.36E-01	CRHR1	Intron	Current study
rs11111272	0.97	G	F	0.29	1.48E-01	IGF1	Intron	Current study
rs11206244	**0.99**	**T**	**F**	**0.32**	**2.78E-01**	**DIO1**	**UTR-3**	**Previous association**
rs1137100	**1.00**	**C**	**R**	**0.28**	**5.17E-01**	**LEPR**	**Missense**	**Previous association**
rs1143627	**0.99**	**G**	**R**	**0.33**	**3.75E-01**	**IL1B**	**5'UTR**	**Previous association**
rs1143634	**0.99**	**T**	**F**	**0.23**	**2.18E-01**	**IL1B**	**Cds - Syn**	**Published association**
rs11591741	1.00	G	R	0.41	6.22E-02	IKKA	Intron	Current study
rs11597086	1.00	C	F	0.41	2.76E-02	IKKA	Intron	Current study
rs11597390	1.00	A	F	0.35	3.74E-02	CPN1	UK	Current study
rs11669332	0.97	T	F	0.07	2.73E-01	AKT2	Intron	Current study
rs11680012	1.00	G	R	0.05	6.25E-01	LRRFIP	Missense	Current study
rs1169300	0.98	A	F	0.32	4.24E-02	HNF1A	Intron	Current study
rs1169302	0.98	C	R	0.45	6.11E-01	HNF1A	Intron	Current study
rs11749561	0.98	T	F	0.47	5.99E-01	NR3C1	UK	Current study
rs1190584	0.99	C	F	0.23	2.35E-01	WDR20	Intron	Current study
rs1205	0.99	T	F	0.33	9.71E-01	CRP	UTR-3	Current study
rs1206034	1.00	A	R	0.34	8.53E-01	LIPE	Intron	Current study

rs12409877	1.00	A	F	0.41	1.35E-01	LEPR	Intron	Current study
rs12626613	1.00	C	R	0.14	9.91E-01	RUNX1	nearGene 5	Current study
rs12750774	0.99	A	F	0.28	4.21E-01	IL6R	Missense	Current study
rs12753193	1.00	G	F	0.40	1.91E-02	LEPR	UK	Current study
rs1417938	1.00	T	R	0.31	1.12E-01	CRP	Intron	Current study
rs1554973	1.00	C	F	0.25	5.86E-01	TLR4	Intron	Current study
rs157580	0.99	C	R	0.40	9.18E-01	TOMM40	UK	Current study
rs16147	0.93	G	F	0.50	1.57E-01	NPY	nearGene 5	Current study
rs1647266	0.99	G	R	0.39	8.25E-03	IFT172	Intron	Current study
rs16892496	0.99	G	R	0.30	6.49E-01	TRHR	UTR-3	Current study
rs16928751	**1.00**	**A**	**F**	**0.12**	**8.16E-01**	**ADIPOR2**	**Cds - Syn**	**Previous association**
rs17295356	1.00	T	F	0.41	6.45E-01	AZGP1	UK	Current study
rs17411031	0.99	G	F	0.27	6.29E-01	LPL	UK	Current study
rs17446593	**0.98**	**G**	**F**	**0.17**	**3.45E-01**	**FOXO1**	**UK**	**Previous association**
rs17446614	1.00	T	R	0.15	8.13E-01	FOXO1	Intron	Current study
rs17689824	1.00	A	R	0.19	8.20E-01	CRHR1	Intron	Current study
rs17700633	1.00	A	F	0.30	2.48E-01	MC4R	UK	Current study
rs17782313	1.00	C	F	0.25	7.79E-01	MC4R	UK	Current study
rs1799964	0.99	C	F	0.20	9.03E-01	TNF	nearGene 5	Current study
rs1800624	0.98	T	R	0.25	3.05E-02	AGER	nearGene 5	Current study
rs1800625	1.00	G	R	0.19	9.84E-01	AGER	nearGene 5	Current study
rs1800896	**1.00**	**G**	**F**	**0.45**	**5.65E-01**	**IL10**	**nearGene 5**	**Published association**
rs1801282	**1.00**	**G**	**F**	**0.13**	**1.04E-01**	**PPARG**	**Intron**	**Previous association**
rs1892534	0.99	A	F	0.40	2.22E-01	LEPR	Rev	Current study
rs1935949	0.99	A	R	0.31	9.97E-01	FOXO3	Intron	Current study
rs1946519	**0.99**	**T**	**R**	**0.40**	**8.45E-01**	**IL18**	**nearGene 5**	**Previous association**

rs2070802	1.00	A	F	0.14	3.85E-01	CNTFR	UK	Current study
rs2071029	0.99	T	R	0.15	7.42E-01	RUNX1	nearGene 5	Current study
rs2229765	0.98	T	R	0.44	8.91E-01	IGFR1	Missense	Current study
rs2239179	0.99	G	F	0.44	8.69E-01	VDR	Intron	Current study
rs2268757	0.99	G	R	0.44	9.74E-02	ACVR2B	Intron	Current study
rs2293152	0.98	C	F	0.40	2.05E-01	STAT3	Intron	Current study
rs2297627	0.99	G	R	0.32	2.63E-01	FOXO1	Intron	Current study
rs2419621	1.00	A	R	0.23	4.68E-01	ACSL5	nearGene 5	Current study
rs2464196	1.00	T	F	0.32	2.59E-02	HNF1A	Missense	Current study
rs2721068	0.99	C	F	0.26	2.35E-01	FOXO1	Intron	Current study
rs2794520	0.99	T	F	0.33	9.82E-01	CRP	UK	Current study
rs2802292	0.99	G	F	0.40	4.38E-01	FOXO3	Intron	Current study
rs2808629	1.00	T	R	0.33	8.95E-01	CRP	UK	Current study
rs281432	**0.99**	**G**	**F**	**0.47**	**7.09E-02**	**ICAM1**	**Intron**	**Previous association**
rs2834650	1.00	A	R	0.10	4.19E-01	RUNX1	Intron	Current study
rs2854464	0.96	G	F	0.28	4.74E-01	INHBC	UTR-3	Current study
rs2960548	**0.95**	**C**	**R**	**0.43**	**1.89E-01**	**APEH**	**Intron**	**Previous association**
rs3088241	**0.98**	**C**	**F**	**0.50**	**3.10E-01**	**HYLS**	**Missense**	**Previous association**
rs3093068	1.00	G	F	0.07	4.69E-01	CRP	UK	Current study
rs3116654	1.00	G	R	0.13	6.78E-01	CRP	UK	Current study
rs3122012	1.00	C	F	0.31	5.12E-02	CRP	UK	Current study
rs3176860	**0.98**	**C**	**R**	**0.38**	**7.04E-01**	**VCAM1**	**Intron**	**Preliminary study**
rs331457	1.00	A	F	0.14	5.73E-01	TRAF6	Intron	Current study
rs3397	**0.98**	**G**	**R**	**0.37**	**5.91E-01**	**TNFRSF1B**	**UTR-3**	**Previous association**
rs35767	1.00	T	F	0.16	4.91E-01	IGF1	nearGene 5	Current study
rs35854772	**0.99**	**T**	**F**	**0.12**	**8.20E-01**	**ADIPOR2**	**Cds - Syn**	**Previous association**

rs361072	0.92	G	F	0.46	3.34E-02	PIK3CB	nearGene 5	Current study
rs3745012	**1.00**	**A**	**R**	**0.26**	**9.37E-01**	**LPIN2**	**UK**	**Preliminary study**
rs3746619	1.00	T	R	0.09	1.18E-01	MC3R	Missense	Current study
rs3791783	0.99	G	R	0.21	6.04E-01	MSTN	Intron	Current study
rs3804099	**0.99**	**C**	**F**	**0.45**	**4.54E-01**	**TLR2**	**Cds - Syn**	**Preliminary study**
rs3856806	**1.00**	**A**	**R**	**0.13**	**4.29E-02**	**PPARG**	**Syn His449His**	**Previous association**
rs4129267	1.00	T	F	0.40	1.81E-01	IL6R	Intron	Current study
rs4131568	1.00	T	R	0.34	1.60E-01	CRP	UK	Current study
rs41423247	0.99	G	R	0.37	5.53E-01	NR3C1	Intron	Current study
rs4149570	**1.00**	**A**	**R**	**0.41**	**1.33E-02**	**TNFRSF1A**	**Intron**	**Previous association**
rs42451	**1.00**	**A**	**R**	**0.27**	**4.08E-01**	**GHRL**	**Intron**	**Previous association**
rs4291	0.96	A	R	0.37	1.86E-01	ACE	nearGene 5	Current study
rs4343	1.00	T	R	0.49	1.48E-01	ACE	Cds - Syn	Current study
rs1544410	0.86	T	R	0.27	UK	VDR	Intron	Current study
rs4855881	**0.99**	**G**	**R**	**0.46**	**5.44E-01**	**APEH**	**Intron**	**Previous association**
rs4946935	0.99	T	R	0.31	7.75E-01	FOXO3	Intron	Current study
rs5010905	0.99	C	F	0.24	9.31E-01	LEPR	UK	Current study
rs4280262	**0.80**	**C**	**F**	**0.12**	**UK**	**LITAF**	**Missense**	**Preliminary study**
rs6136	**0.97**	**G**	**R**	**0.09**	**2.69E-01**	**SELP**	**Missense**	**Previous association**
rs6198	1.00	C	R	0.17	6.14E-01	NR3C1	UTR-3	Current study
rs6220	0.97	C	R	0.29	1.56E-01	IGF1	UTR-3	Current study
rs7136446	0.97	G	R	0.39	2.41E-02	IGF1	Intron	Current study
rs7187438	**0.98**	**G**	**R**	**0.36**	**9.88E-02**	**TSC2**	**Intron**	**Previous association**
rs7209436	0.97	A	R	0.43	2.69E-01	CRHR1	Intron	Current study
rs7310409	0.99	T	R	0.41	3.60E-03	HNF1A	Intron	Current study
rs731236	0.99	C	F	0.40	2.96E-01	VDR	Cds - Syn	Current study

rs744166	1.00	G	R	0.41	4.52E-01	STAT3	Intron	Current study
rs7539471	0.98	A	F	0.31	1.09E-02	LEPR	UK	Current study
rs767455	**0.98**	**C**	**F**	**0.42**	**7.08E-02**	**TNFRSF1A**	**Cds - Syn**	**Previous association**
rs767870	1.00	C	F	0.15	9.15E-01	ADIPOR2	Intron	Current study
rs769449	0.99	A	F	0.11	6.48E-01	TOMM40	Intron	Current study
rs780106	1.00	C	F	0.39	1.15E-02	IFT172	Intron	Current study
rs7832552	0.98	A	R	0.30	6.17E-01	TRHR	Intron	Current study
rs8069645	1.00	G	F	0.27	8.79E-01	STAT3	Intron	Current study
rs817529	**1.00**	**C**	**R**	**0.38**	**7.83E-01**	**LTBP1**	**Intron**	**Previous association**
rs863013	0.98	A	R	0.32	3.93E-01	CRP	UK	Current study
rs9402571	0.98	G	F	0.18	9.75E-01	SGK1	Intron	Current study
rs972936	1.00	T	R	0.27	1.07E-01	IGF1	Intron	Current study
rs976881	**0.97**	**T**	**R**	**0.32**	**2.57E-01**	**TNFRSF1B**	**Intron**	**Previous association**

Table legend: List of polymorphisms following data pruning strategies as mentioned in methods section are retained for association analysis. CR, SNP call rate; MA, minor allele; MAF, minor allele frequency; Gene, gene symbol; Source of SNP, the SNPs (rs#) considered from a previous association study(207), published association(197, 200, 209) and a preliminary study(755) are replicated and shown in bold. The remaining SNPs are newly reported in this study. The sequencing strand for genotyping is indicated and the minor allele shown is based on the measured frequencies in our study population (predominantly of Caucasian origin) and is thus independent of the strand sequenced in the genotyping assay.

6.4.2 Patient demographics

General characteristics of the genotyped patient population are presented (Table 6.3). Average age of the cohort at diagnosis was 65 ± 13 years (mean ± SD). The majority were diagnosed with stage III or IV cancer. At diagnosis, average WL was 6 ± 9% with a mean body mass index (BMI) of 25 ± 5. Of the 751 patients with CT scans for the assessment of muscularity 47% had LM defined by the specified cut offs. There were no significant differences in age, stage of disease, pre-diagnosis BMI and percentage WL between patients who had CT scans suitable for the measurement of muscularity and the entire cohort (Table 6.3).

Table 6.3 - Patient demographics

Patients were recruited (2004 – 2012) from: NHS Lothian, UK; Cross Cancer Institute, Edmonton, Canada; McGill University Health Centre, Montreal, Canada; Palliative Research Centre, Norwegian University of Science and Technology (NTNU), Norway; Cantonal Hospital, St Gallen, Switzerland; and Department of Medical Oncology, University Hospital of Larissa.

	New n=545	Old n=731	All n=1276
Age (years) †	66 ± 11	66 ± 11	65 ± 13
Range	(22-91)	(27-97)	(22-97)
Sex			
M	333	446	779 (61)
F	212	285	497 (39)
Tumour type			
Oesophageal or Gastric	49	356	405 (32)
Pancreatic	50	108	158 (13)
Lung	318	232	550 (43)
Other	128	35	163 (12)
Stage			
I	44	33	77 (6)
II	30	80	110 (9)
III	229	435	664 (52)
IV	242	183	425 (33)
Body mass index (kg/m^2)†	25 ± 5	24 ± 7	25 ± 5
Range	(14-59)	(13-47)	(13 – 59)
Percentage weight loss†	5 ± 7	7 ± 10	6 ± 9
Patients with CT scans	-	-	751
Skeletal muscle index cm^2/m^2†§			
M	-	-	49 ± 9
F	-	-	41 ± 7

Values are number of patients with percentages in parentheses unless indicated otherwise; †values are mean ± SD. Characteristics were measured at first presentation to a surgical or oncology clinic.

§Skeletal muscle index calculated as lumbar total muscle cross-sectional area (cm^2)/height (m)^2.

6.4.3 Weight loss phenotype: Stage 2 analysis of previously identified SNPs in newly recruited cohort (n=545)

Table 6.4 lists the detailed results for previously identified SNPs significantly associated with cancer cachexia in the newly recruited patients classified according to WL alone. Two SNPs were validated in this new cohort, one being found on chromosome 1 in the SELP gene and was present at all levels of WL (rs6136). The SELP gene encodes for p-selectin, a molecule which functions as a cell adhesion molecule (CAM) in response to inflammation on the surfaces of activated endothelial cells (756). The second SNP (rs4149570), found in the TNFRSF1A gene on

chromosome 12 was found to be significant at >15% WL. This gene encodes for one of the major receptors for tumour necrosis factor-α. The downstream effects of this receptor are activation of the transcription factor NF-κB to mediate apoptosis, and function as a regulator of inflammation (757).

Table 6.4a - Genes with variants significantly associated with cancer cachexia from previous stage 1 analysis in newly recruited patients classified according to weight loss alone

Weight loss >15%. Number affected: 62/545 (11.3%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
TNFRSF1A	rs4149570	T	0.628 (0.402-0.980)	0.041	0.044
SELP	rs6136	C	0.331 (0.117-0.933)	0.036	0.054

Table 6.4b - Weight loss >10%. Number affected: 129/545 (23.6%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
SELP	rs6136	C	0.332 (0.160-0.692)	0.003	0.003

Table 6.4c - Weight loss >5%. Number affected: 239/545 (43.7%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
SELP	rs6136	C	0.543 (0.334-0.881)	0.013	0.014

6.4.4 Weight loss phenotype: Stage 1 analysis with new candidate SNPs in combined cohort (n=1276)

Table 6.5 lists the detailed results for newly identified SNPs significantly associated with cancer cachexia in all patients classified according to WL alone. In total, 12 SNPs had significant associations with various cachexia phenotypes based on increasing severity of WL. Two of these SNPs (rs1935949 and rs4946935) found within chromosome 6 in the Forkhead box O3 (FOXO3) gene were associated with WL of increasing severity (>5% and >10%) and one SNP (rs2297627) found in the Forkhead box O1 (FOXO1) gene was associated with WL>10%. FOXO1 and FOXO3 belong to the forkhead family of transcription factors. These transcription factors share the ability to be inhibited and translocated out of the nucleus on phosphorylation by proteins such as Akt/PKB in the PI3K signalling pathway (758). The FOXO proteins influence apoptosis through upregulation of genes necessary for cell death (759).

Table 6.5a - Genes with variants significantly associated with cancer cachexia in patients classified according to weight loss alone

Weight loss >15%. Number affected: 199/1276 (15.6%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
LEPR	rs5010905	C	1.547(1.136-2.108)	0.006	0.003
IGF1	rs35767	T	0.621 (0.426-0.905)	0.013	0.014
CPN1	rs11597390	A	1.309 (1.009-1.698)	0.042	0.038

Table 6.5b - Weight loss >10%. Number affected: 382/1276 (29.9%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
IGF1	rs35767	T	0.677 (0.507-0.905)	0.008	0.008
FOXO3	rs1935949	T	1.31 (1.05-1.635)	0.017	0.012
FOXO3	rs4946935	A	1.281 (1.026-1.599)	0.029	0.027
LEPR	rs12409877	A	0.796 (0.641- 0.988)	0.038	0.040
FOXO1	rs2297627	C	0.795 (0.638-0.990)	0.040	0.049
TOMM40	rs157580	G	1.24 (1.006-1.529)	0.044	0.050

Table 6.5c - Weight loss >5%. Number affected: 633/1276 (49.6%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
IFT172	rs1647266	C	0.788 (0.665-0.933)	0.006	0.006

IFT172	rs780106	C	0.803 (0.679-0.950)	0.011	0.009
FOXO3	rs1935949	T	1.244 (1.035-1.495)	0.012	0.022
ACVR2B	rs2268757	C	1.219 (1.032-1.44)	0.020	0.024
TLR4	rs1554973	C	1.238 (1.014-1.512)	0.036	0.028
FOXO3	rs4946935	A	1.227 (1.021-1.474)	0.029	0.034

6.4.5 Low muscularity phenotype: Stage 1 analysis with all candidate SNPs in combined cohort with CT scans (n=751)

Table 6.6a lists the detailed results for all SNPs significantly associated with cancer cachexia in all recruited patients (n=751) classified according to LM alone. In total, 5 SNPs were associated significantly with the cachexia phenotype based on levels of muscularity: (i) rs4291 in chromosome 17 in the angiotensin converting enzyme (ACE) gene; this gene has been associated with muscle function and metabolism(172); (ii) rs10636 in chromosome 16 in the metallothionein 2a gene; this gene has been shown to be involved in zinc dyshomeostasis which may contribute to cancer cachexia(703); (iii) rs1190584 in chromosome 14 in the WDR20 gene; this gene encodes a WD repeat-containing protein that functions to preserve and regulate the activity of the USP12-UAF1 deubiquitinating enzyme complex; (iv) rs3856806 in the peroxisome proliferator-activated receptor gamma (PPARG) gene in chromosome 3 which has been demonstrated to be involved in fatty acid and glucose metabolism(760); and (v) rs3745012 in chromosome 18 in the lipin 2 (LPIN2) gene; this gene represents a candidate gene for human lipodystrophy, characterised by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance.

6.4.6 Low muscularity plus weight loss phenotype: Stage 1 analysis with all candidate SNPs in combined cohort (n=943)

Table 6.6b lists the detailed results for all SNPs associated significantly with cancer cachexia classified according to LM + WL >2% in all recruited patients. The analysis compared those with the LM + WL >2% phenotype against those without in the entire cohort. rs12409877 is in the leptin receptor (LEPR) located on chromosome 3, LEPR binds leptin and is involved in adipose tissue regulation(761). rs2268757 is located in the activin receptor type-2B (ACVR2B) gene on chromosome 3, ACVR2B is a high affinity activin type 2 receptor which mediates signalling by a subset of TGF-β family ligands including myostatin, activin, GDF11 and others(762). SNPs in the tumour necrosis factor (TNF) (rs1799964) and ACE (rs4291) genes were also significantly associated with the phenotype.

Table 6.6a - Genes with variants significantly associated with cancer cachexia in patients classified according to low muscularity alone

LM. Number affected: 356/751 (47.4%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
ACE	rs4291	T	1.326 (1.058-1.662)	0.01431	0.017
MT2A	rs10636	C	1.342 (1.053-1.711)	0.01736	0.018
WDR20	rs1190584	C	0.729 (0.563-0.943)	0.01621	0.023
PPARG	rs3856806	T	1.353 (1.003-1.824)	0.04782	0.046
LPIN2	rs3745012	T	0.776 (0.604-0.996)	0.04646	0.047

Table 6.6b - Genes with variants significantly associated with cancer cachexia in patients classified according to low muscularity and weight loss >2% compared with those who do not

LM and weight loss >2%. Number affected: 214/943 (22.7%)

Gene	SNP	Risk allele	OR (95%CI)	*p*-Value	Permutated *p*
LEPR	rs12409877	A	0.674 (0.526-0.865)	0.002	0.002
ACVR2B	rs2268757	C	1.406 (1.126-1.757)	0.003	0.002

TNF	rs1799964	C	1.435 (1.093-1.885)	0.010	0.010
ACE	rs4291	T	1.313 (1.039-1.659)	0.022	0.025

6.4.7 Combining genes with functional similarity according to gene ontology

Table 6.7 lists the phenotypes for which candidate gene groups associated with specific cancer cachexia phenotypes. SNPs in groups of genes involved in appetite regulation, cell adhesion, cell membrane structure and function, and signal transduction were associated with the phenotype WL >10%. Only SNPs in the group of genes involved in cell adhesion were significant with increasing WL. SNPs in groups of genes involved in lipid metabolism, appetite regulation, signal transduction and glucocorticoid signalling were associated with the phenotype LM and WL >2%. No SNPs in groups of genes were found to be significant with all other phenotypes.

Table 6.7 - Candidate gene groups associated with cancer cachexia phenotypes

Phenotype	Candidate gene group function	Number of genes*	Number of SNPs	p-Values
Weight loss >5%	N/A	N/A	N/A	N/S
Weight loss >10%	Appetite regulation Cell adhesion Cell membrane structure and function Signal transduction	8 12 32 51	21 17 66 110	0.0041 0.0054 0.0370 0.0380
Weight loss >15%	Cell adhesion	12	17	0.0193
LM	N/A	N/A	N/A	N/S
LM + Weight loss>2%	Appetite regulation Signal transduction Glucocorticoid signalling Lipid metabolism	8 51 4 15	21 110 6 38	0.0138 0.0233 0.0337 0.0388

*The genes in each candidate gene group are listed in Table 6.1

N/A = Not applicable

N/S = Not significant

6.4.8 Transcriptomic analysis

Table 6.8 lists the results from correlation and t-test analysis between phenotypes and gene transcript level for the genes that showed significant associations with any of the cachexia phenotypes. Expression of ACVR2B, FOXO1 and 3, LEPR, PPARG, TLR4, and TOMM40 transcripts was significantly associated with different levels of SMI or WL ($P<0.05$). Specifically, these were all negatively correlated with muscularity

(Figure 6.1). FOXO1 and 3 and TOMM40 were the only genes significantly correlated with WL; these were correlated negatively with WL.

Table 6.8 - Results from correlation and t-test analysis between patient characteristics and rectus abdominis muscle gene transcripts for selected genes[1]

Probe Name	Gene Symbol	Correlation between probes within genes [2]	Correlation with SMI, cm²/m² (n=102)[3]	Correlation with % weight loss, %/100d (n=86)[3]	FD[4]	T-test of SMI values for patients with high vs. low probe expressionp-value[5]	T-test of % weight loss/100d values for patients with high vs. low probe expression p-value[5]
A_23_P109950	ACVR2B	0.94	-0.21[a]	-0.03	3.9	**0.04**	0.86
A_24_P231132	ACVR2B		-0.24[a]	-0.09	3.0	**0.03**	0.17
A_23_P151426	FOXO1	0.71	-0.22[a]	-0.23[a]	4.2	**0.01**	**0.04**
A_24_P22079	FOXO1		-0.39[a]	-0.43[a]	3.5	**< 0.01**	**< 0.0**
A_23_P345575	FOXO3	0.97	-0.31[a]	-0.29[a]	2.9	**< 0.01**	**0.01**
A_32_P102062	FOXO3		-0.33[a]	-0.28[a]	3.1	**< 0.01**	**0.01**
A_23_P161135	LEPR		-0.26[a]	0.00	3.5	**< 0.01**	0.31
A_23_P252062	PPARG		-0.16	0.02	3.8	**0.03**	0.49
A_23_P60306	TLR4	0.60 to 0.85	-0.18	-0.07	2.1	**0.02**	0.19
A_24_P69538	TLR4		-0.10	-0.01	2.1	**0.03**	0.95

A_32_P66881	TLR4	-0.19	-0.15	2.0	**0.04**	0.29
A_23_P153266	TOMM40	-0.30 [a]	-0.26 [a]	1.8	**< 0.01**	**< 0.01**

SMI, skeletal muscle index; FD, fold difference.

[1]The following gene probes did not show significant Pearson correlation or significance for the t-test analysis: ACE probes (A_23_P371777, A_23_P38235, A_24_P365129), ACVR2B probe (A_32_P134209), CPN1 probe (A_23_P98147), IFT172 probe (A_23_P406135), IGF1 probes (A_23_P13907, A_24_P304419, A_24_P304423, A_24_P398572), LEPR probe (A_24_P231104), LPIN2 probes (A_23_P208055,

A_24_P301557), MT2A probes (A_23_P106844, A_23_P252413, A_24_P361896), SELP probe (A_23_P137697), TNF probes (A_23_P376488, A_24_P50759), TNFRSF1A probes (A_23_P139722, A_24_P364363), TOMM40 probes (A_24_P15754, A_24_P178093), WDR20 probes (A_23_P205255, A_24_P184769, A_24_P162412)

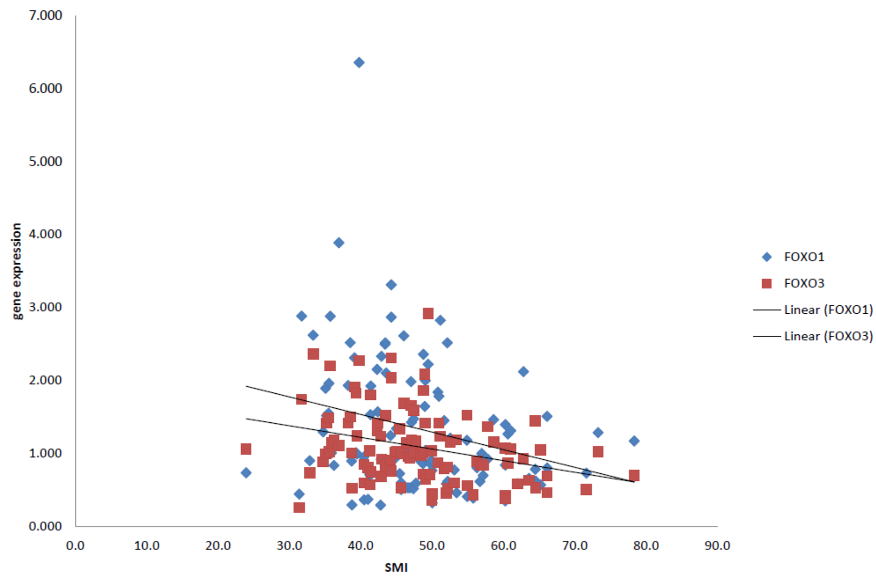
[2]Pearson correlation analysis was conducted only for genes with multiple probes.

[3] Pearson correlation analyses were conducted to identify linear relationships between gene probe intensities and SMI or weight loss. Note that not all 134 patients had both SMI and weight loss information available and therefore the number of patients for the SMI and weight loss correlation analysis were 102 and 86, respectively. [a]Pearson correlation p-value < 0.05

[4] Fold change = average high expressors / average low expressors

[5] The average sample size for the T-test comparing the SMI values for patients with high versus low probe expression was 35 and 33, respectively. The average sample size for the T-test comparing the % weight loss/100d values for patients with high versus low probe expression was 30 and 29, respectively. These sample sizes differed slightly from test to test as not all patients had computing tomography scans for SMI measurements and not all patients had WL values in their clinical charts.

A



B

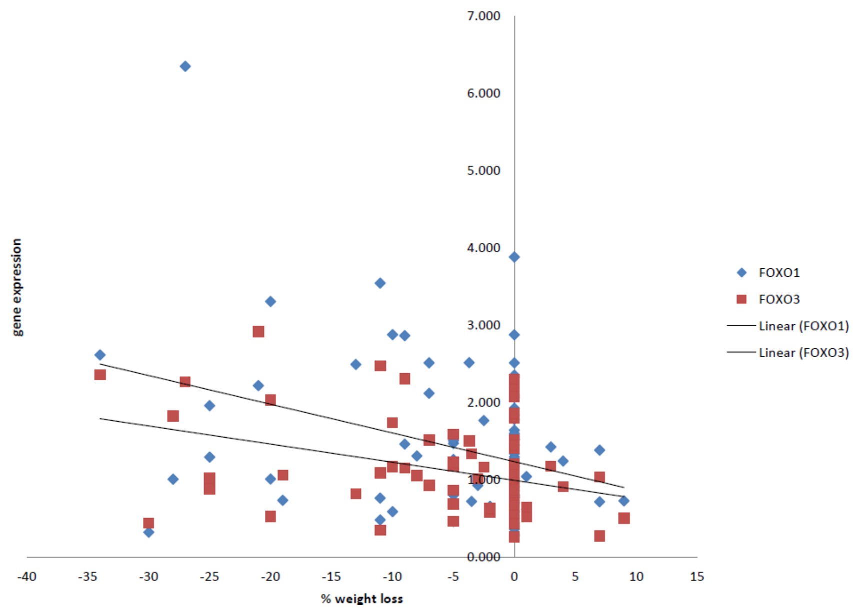


Figure 6.1 – Graphical representation of the FOXO1 and FOXO3 gene expression compared to SMI (A) and % WL (B). Expression FOXO1 and FOXO3 transcripts was significantly associated with different levels of SMI or WL (P<0.05). Specifically, these were all negatively correlated.

6.5 Discussion

6.5.1 Associations with different cachexia phenotypes

In keeping with the prior study (207), this Chapter has confirmed in a more adequately sized validation cohort (Stage 2, n=545) that patients who carry the C allele of the rs6136 SNP in the SELP gene are at a reduced risk of developing cachexia defined by WL (>5%, >10% or >15%), (Table 6.4). This was also confirmed recently in a study of chemo-naïve patients with locally advanced or metastatic pancreatic cancer where the presence of the C allele of rs6136 was associated with a reduced risk of developing cachexia (as defined by WL >5% or >2% in those with a BMI <20kg/m^2 (207)) in both the discovery (n=151) and validation (n=152) cohorts (206). It is interesting that the same study identified patients with the GG allele of rs1130233 on the AKT1 gene to also be at reduced risk of cancer cachexia. In our prior study with a larger sample size, a different SNP on the AKT1 gene (rs1130214) was evaluated and found not to associate with the WL phenotype (207). The G-allele of the rs1800796 polymorphism in the IL6 gene has been shown to be associated with increased susceptibility to cachexia and decreased survival time of stage II and III Chinese pancreatic cancer patients (202). There was not an association with rs1800795, which shows a weak linkage disequilibrium with the rs1800796 and neither did the aforementioned recent study of chemo-naïve patients with locally advanced or metastatic pancreatic cancer (206). In the present study, a number of other IL6 polymorphisms were included that also failed to show association. These differing findings may, in part, be due to insufficient representation of pancreatic cancer patients in our cohort or the differences in ethnicity.

For the WL phenotype, the present study examined 92 new SNPs in a stage 1 analysis that included 1276 cancer patients, the largest sample size to date in an association study for cancer cachexia. Twelve new candidate SNPs were identified (Table 6.5). Six of these SNPs are associated with muscle metabolism in five genes (IGF1, CPN1, FOXO1, FOXO3, and ACVR2B), three are associated with adipose tissue metabolism in two genes (LEPR and TOMM40 (APOE on the reverse strand)), two with corticosteroid signalling in one gene (IFT172 (GCKR on the reverse strand)) and one with the immune response in one gene (TLR4). Two polymorphisms (rs1935949 and rs4946935) in the

gene encoding for FOXO3 were consistently associated with WL of increasing severity (Table 6.5). On the basis that WL is a continuum in the cachectic process, the observation that both SELP and FOXO3 associate with the highest degrees of WL suggests that these genetic signatures may be of particular significance.

For the phenotype based on LM alone, five SNPs were identified (Table 6.6a). Two of these SNPs are associated with muscle metabolism in two genes (ACE and WDR20), two with adipose tissue metabolism in two genes (PPARG and LPIN2), and one with metallic ion metabolism in one gene (MT2A). In the present study, when patients were classified as cachectic according to WL and LM, four SNPs were identified (Table 6.6b). Two of these SNPs are associated with muscle metabolism in two genes (ACVR2B and ACE), one with fat metabolism in one gene (LEPR), and one with cytokine production in one gene (TNF). The observation that most of the identified genes are associated with muscle and adipose tissue metabolism highlights the importance of these processes in the progression of cancer cachexia. Skeletal muscle wasting is a signature event in cancer cachexia and could be associated with increased fatty infiltration (396, 763), therefore mechanisms effecting the functions of these genes may be of particular significance. It would be attractive to assign specific functional significance to some of the genetic signatures so identified. For example ACVR2B decoy receptors have been shown to abrogate muscle loss and prolong survival in several murine models of cancer cachexia (145). However, replication of the present findings along with a genome-wide approach would be needed in parallel with functional studies (see below) to resolve this issue further.

6.5.2 Gene group analysis

The two dominant mechanisms of weight loss in cancer patients are anorexia leading to reduced food intake and abnormal metabolism (11). Appetite regulation was found to be most significantly associated with the cachexia trait WL >10% ($p=0.0041$). There has been some evidence to date that negative regulators of appetite such as PYY are elevated in cachexia(764). Regarding metabolic change, lipid metabolism was significantly associated with the cachexia trait LM and WL >2% ($p=0.0138$). Fatty infiltration (myosteatosis) has been associated with cancer cachexia and reduced survival

(396, 763). The glucocorticoid signalling pathway was also found to be associated with cachexia trait LM and WL >2% (p=0.0337). Glucocorticoids and associated signalling pathways have been implicated in accelerating protein degradation in muscle (765). Glucocorticoids are thought to act through a permissive effect on the upregulation of messenger RNA and the subsequent synthesis of components of the ubiquitin proteasome pathway in muscle. Glucocorticoids may also inhibit protein synthesis and promote gluconeogenesis, and suppress glucose and amino acid muscle uptake by inhibiting cellular transporters(766).

6.5.3 Differential gene expression signatures in human muscle and their association with the phenotypes of cachexia

It has been shown previously that the presence of cancer cachexia is associated with the transcriptional regulation of about 1800 genes in human skeletal muscle (89). It was therefore of interest to see if the transcript level of the genes associated with cachexia in the present study were altered in a separate cohort of cancer patients with or without cachexia. Indeed, there was a strong concordance between a proportion of the selected genes and either the level of WL or muscularity (Table 6.8). FOXO1 and FOXO3 are good examples: SNPs in both genes were identified to associate with the WL phenotype (Tables 6.5b and c) and transcript levels of both showed a correlation with WL (Table 6.8). These transcription factors are key in the pro-inflammatory driven up-regulation of the ubiquitin-proteasome pathway, but also act as a negative regulator of the anabolic Akt-mTOR pathway (329, 758). Inhibition of FOXO transcriptional activity prevents muscle fibre atrophy during cancer cachexia and induces hypertrophy (95). It is therefore plausible that variants of the FOXO genes are key determinants of the muscle loss component of cachexia syndrome.

The present analysis was not of a genome-wide nature and therefore genetic variants with much greater functional significance may have not have been examined. Equally, the true functional significance of any individual SNP for cancer patients is mostly unknown. It may, therefore, be better to consider the genetic associations identified in the present analysis as genetic signatures or biomarkers associated with the cachexia

syndrome. Interestingly, 17 of the 19 SNPs reported as showing significant associations with the cachexia phenotypes are in intronic, 3', or 5' UTRs. The purpose was to explore the trends and probe into the potential functional impact of the loci as SNPs in this study are potentially proxy to the more causal variants (not yet captured in the region) which may also have an influence on gene expression; as such the probe position in the expression array and the SNP position are not the same. Extrapolation to a SNP under investigation being an expression quantitative loci (eQTL) is premature. The SNP identified may in some cases also affect gene expression signatures not addressed herein (as in cis- and trans acting eQTLs). The correlation pattern (albeit, low to modest) observed is still encouraging since the trends reported here for a SNP loci and gene expression are within the scope of known cachexia literature. Further replication of the present findings and fine mapping of the loci may offer insights. Independent of the functional significance of the polymorphisms, the SNP signatures serve as heritable determinants for cancer cachexia; the polygenic nature of the phenotype warrants identification of more susceptibility variants to enable stratification of patients for cancer cachexia risk.

Equally, for those genes for which no strong relationship was found between gene expression and patient characteristics it is important to consider that these may not be transcriptionally regulated. For the various humoral mediators (ACE, TNF, IGF1), it may be that the concentration in blood is important rather than local expression in tissues since tissue-specific expression may be transient for some genes but the activation of the signal transduction cascade could be the largest prevailing effect. For instance local expression of the ligand may be a small part of a bigger picture where several tissue sources of ligand, a variety of receptors, soluble receptors and binding proteins may play in to the actual response (e.g., TNF and IGF-1).

6.5.4 Limitations

The present investigation is part of a series undertaken by our international consortium which has over time expanded DNA biobanking and cachexia phenotyping from 775 to 1276 patients. While these represent the largest candidate gene association studies done to date in relation to cancer cachexia, they have the general limitation of the candidate

gene approach. The assembly of much larger data sets and samples in order to conduct genome wide association studies is awaited. The current study combined different tumour types in the analysis. As the hypothesis states, for any given cohort it is impossible to predict who will develop cancer cachexia and who will not, therefore two different patients with the same stage of tumour may have vastly different clinical presentations in terms of cachexia, based on this the tumour types were combined. With the recruitment of more patients it may be possible to reduce this heterogeneity.

It is important to establish that the prevalence of LM in the present cancer cohort is in excess of that which would be observed in the normal age-matched population. The prevalence of LM/sarcopenia in age-matched subjects living in the community varies according to the precise definition and methodology used, but is reported to be between 1-29% (767). The prevalence of LM that was observed in the cancer cohort in this study was 44% for males and 52% for females. Thus, the gene associations established with LM represent associations with a level of muscularity at least partly independent of age, sex or stature.

Selection of the genes and SNPs may be biased towards genes with a known potential influence on WL. Therefore genes used for other processes associated with cachexia such as muscle and lipid regulation are under-represented and potential associations may have been missed in these key regulatory pathways. As many of these genes have been recently discovered there is paucity of functional significance. As far as the muscle and lipid regulation phenotypes are concerned, the present study begins a journey into defining phenotypes based on these novel measurements. Other independent studies need to be conducted to address the muscle loss phenotype and its associated genes and pathways. It is also important to recognise that the understanding of the biology of cachexia is evolving rapidly and that factors such as GDF-15 (MIC-1) that might now be considered as potentially important(768), were not included in the present study.

6.5.5 Conclusions

The candidate gene SNP analysis offers the advantage that it is hypothesis driven and the identified associations are easily explained due to compelling biological rationale.

However, the limitations of this approach are that the role of hitherto unexplored genes and pathways that otherwise contribute to the trait under investigation are missed. Issues surrounding phenotype complexity are addressed in part in this study and conducting a genome wide association study (GWAS) using high density of markers on the genome would help relate the overlap of SNPs/pathways to the phenotypes of interest. The consensus definitions for phenotypes may evolve in an iterative manner from the cumulative wisdom from candidate SNPs, GWAS and the current definitions available for cachexia.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE WORK

Cancer cachexia significantly impairs quality of life and response to treatment and is associated with increasing morbidity and mortality. This thesis set out to clinically classify cancer cachexia based on phenotypic correlates in human skeletal muscle to provide accurate phenotyping for a genetic study into the potential predisposition to developing cancer cachexia to aid with identification of biomarkers to screen for the syndrome.

7.1 Application of CT scanning for body composition analysis

One of the main themes running through this thesis is the use of CT scan imaging to derive measures of body composition to aid with accurate phenotyping. CT scans are increasingly used as research tools for body composition analysis in a number of conditions including cachexia (396, 397, 746). CT scans allow segmentation of specific tissues and provide direct measures of tissue cross sectional area (CSA) (cm^2). Single slice quantification of muscle and adipose tissue and specific muscle groups using CT has been shown to be representative of true muscle and adipose CSA of cadaver axial sections (393, 769). Research utilising CT scans has revealed that variation in individual tissue compartments may confer specific risks upon particular patient groups. Sarcopenia (generally defined as an absolute muscle mass > 2 standard deviations below expected for young adults) is associated with functional impairment, disability and survival (350, 770-773). It has been shown that sarcopenic obesity is an adverse prognostic factor associated with reduced survival in several cancer types (12, 28). This has led to suggestion that cancer cachexia should be defined by incorporating a level of muscularity in the diagnostic algorithm. CT scanning is useful as it forms part of a patient's standard of care therefore making it readily accessible. A number of studies address the repeatability of cross sectional imaging in body composition (774-778) and report good agreement between repeated measurements using CT for both fat and muscle. Segmentation of different tissue types using CT is achieved using defined Hounsfield Units (HU) for specific tissues in conjunction with manual correction. In this context variations in the image analysis software appear to exert little influence on measurements of CSA (779, 780). In many publications data are reported using directly determined units

of tissue cross-sectional area (cm^2) from a single image (781, 782). However, several authors have worked to find the single image which is most representative of whole-body organ volumes (i.e. for muscle and adipose tissues) (783, 784). The intention of this has been to estimate values for total body fat, fat free mass, skeletal muscle mass or regional volumes such as thigh muscle or visceral adipose, while limiting the time taken for analysis. As a result cross sectional data are often used as a surrogate for these measures, since they are linearly related to whole body or regional measures. Regional analysis of fat and lean tissue (e.g. L3 level) are highly correlated to corresponding whole body compartments and can provide precise quantification of specific adipose tissues, skeletal muscles and organs (28, 393, 395, 397, 746, 785).

CT scanning for body composition has its limitations. CT scans involve ionising radiation in addition to the accessibility of the measurements to the researcher and clinician. Patient positioning, technical errors, movement, slice selection and image interpretation including identification of specific muscles (previously described) and also within the abdominal cavity where the peritoneum may be incorrectly marked (786) or intestinal contents identified as anatomical adipose (787) all are factors when using CT scans to quantify body composition. However by understanding the limitations and potential sources of error when using CT imaging for measurement of body composition the technique can reliably measure muscle and fat distribution in the trunk and can discriminate between the intra-abdominal organ and muscle component of fat free mass. Using CT imaging compares well with other body composition measures and has the significant advantage of being readily available in patients with cancer.

One of the main findings of this thesis, outlined in the study in Chapter 4, is for the first time, the muscle protein content of analysed CT scans has been measured and has shown to be reduced. In the study reported in Chapter 4, when compared with non-cachectic patients, muscle protein content was reduced significantly (approximately 13%) if patients were classified as cachectic by either >5% WL (p=0.015) or LM + >2%WL (p=0.035), and by 10% in patients with >10% WL. Protein content expressed in relation to wet weight of muscle has been shown to decrease progressively (in excess of 50%) in the gastrocnemius muscle of mice bearing the MAC-16 tumour (257). This suggests that not only is there loss of fibre diameter, but that the quality of the fibre is altered with loss of either sarcoplasmic or myofibrillar protein. Such changes in fibre composition may

contribute to the reduced muscle mechanical quality (force per unit cross-sectional area) observed in human cancer cachexia (213). Whist using CT scans to derive measures of body composition is useful, caution has to be exercised because these are only volume measures.

7.2 Clinical Classification of Cancer Cachexia by deriving Phenotypic Correlates in Human Skeletal Muscle

The international consensus on the classification of cancer cachexia (11) suggested that diagnostic criteria should take into account not only that weight loss is a signal event of the cachectic process but that the initial reserve of the patient should also be considered (either low BMI or low level of muscularity). Although the latter concept has some validation in terms of clinical risk, there has been no evaluation of the biological correlates in terms of changes within skeletal muscle itself. The study reported in Chapter 4 demonstrated when patients were classified as cachectic or not according to ≥ 5% weight loss there was no significant difference in whole body muscularity (FFMI) or muscle fibre CSA. In contrast, when patients were classified according to low muscularity and ≥ 2% weight loss, FFMI was decreased and fibre cross sectional area was also significantly reduced. Such findings demonstrate that heterogeneity in relation to low muscularity and fibre atrophy may be reduced according to the clinical definition of cachexia. This finding may be important especially when considering inclusion criteria for clinical trials that aim to test the efficacy of drugs targeted at reversal of muscle wasting (fibre atrophy) in cancer patients. In cachexia there is conflicting evidence as to whether there is selective loss of fibre type. No evidence for selective loss of fibre type was found in the study reported in Chapter 4. This is consistent with the literature as it is currently not entirely clear which type of fibres are affected in human cancer cachexia. However, in patients with oesophago-gastric cancer cachexia early loss of all MyHC isoforms has been reported. The activity patterns of a muscle are also key in determining phenotype. If muscle cells

are recruited infrequently they develop into fast/glycolytic units whereas if they are recruited more often, they form slow/oxidative units. One limitation of the study reported in Chapter 4 is that there is no functional data included which is clearly an important variable in the development of cancer cachexia.

The study reported in Chapter 4 looked at muscle protein, DNA, and RNA content in cancer cachexia which has seldom been studied in humans before. When compared with non-cachectic patients, muscle protein content was reduced significantly (approximately 13%) if patients were classified as cachectic by either >5% WL ($p=0.015$) or LM + >2%WL ($p=0.035$), and by 10% in patients with >10% WL. This suggests that not only is there loss of fibre diameter, but that the quality of the fibre is altered with loss of either sarcoplasmic or myofibrillar protein. Such changes in fibre composition may contribute to the reduced muscle mechanical quality (force per unit cross-sectional area) observed in human cancer cachexia (213). In Chapter 4, RNA content was unaltered in cachectic patients (classified either with >5% WL or LM + >2% WL) compared with non-cachectic patients. This is consistent with a recent observation that protein synthesis is unaltered (80). The study reported in Chapter 4 demonstrated DNA content was increased by >50% with >5% WL but decreased by 40% in patients with LM. Because mature myofibre nuclei are thought to be mitotically inactive, increased DNA content in skeletal muscle cells suggests activation of satellite cells or infiltration by other cell types such as inflammatory cells or adipocytes. In the LM group, the decrease in DNA may be due to pre-existing age-related sarcopenia or other causes of muscle atrophy (e.g. immobilisation) and may relate to muscle specific apoptosis and reduction in cell number in keeping with a reduction in muscle mass on CT scanning. The diametrically opposite changes in muscle DNA content dependent on whether patients are classified according to weight-loss or low muscularity again underpin the potential diverse mechanisms whereby older cancer patients may develop a low level of muscularity. The issue of whether nuclear domain size is reduced in cancer cachexia remains to be resolved. In particular, whether apoptosis in skeletal muscle is increased in cancer cachexia and the degree to which DNA content is maintained or not via a compensatory increase in myonuclear number (possibly via satellite cell nuclei incorporation) is not known. Features of cachexia such as hypogonadism (resulting in low testosterone) or systemic inflammation (associated with high IL-6) could influence such regenerative capacity. In Chapter 4 RNA / DNA was

shown to be altered in the cachectic patients (independent of definition) compared with the non- cachectic patients. This may be due to the interplay of the mechanisms described above. Autophagy proteins (ATG) 5, 7, 12, and beclin 1 were also studied. These proteins are necessary for autophagy due to their role in autophagosome elongation. When patients were classified according to >10% WL, Beclin and ATG5 protein levels were significantly increased in cachectic patients when compared with non-cachectic patients. Further research needs to be done to validate these findings as the autophagy pathway may be an important mechanism in the development of cancer cachexia. Systemic inflammation is thought to be a major mediator of cancer cachexia. However, the relationship between inflammation in the systemic compartment versus muscle and the relationship of either to muscle loss in humans is not clear. Although The study reported in Chapter 4 presented evidence for systemic inflammation in a proportion of patients, no significant difference was found in the levels of phospho-NFkB or phospho-STAT3 across any of the definitions of cachexia or in those with or without evidence of systemic inflammation.

It is possible that inflammatory mediators have their main effects on muscle atrophy via central mechanisms mediated via the CNS. It has been suggested that binding of myostatin to the ACVR2B receptor results in the phosphorylation of two serine residues of SMAD2 or SMAD3. This leads to the assembly of SMAD2/3 with SMAD4 to the heterodimer that is able to translocate to the nucleus and activate transcription of target genes. One of the known downstream targets of SMAD signalling is MyoD, a transcriptional factor that is involved in skeletal muscle development and takes part in the repair of damaged skeletal muscle. When patients were classified as cachectic according to >5% WL, SMAD3 protein levels were significantly increased in cachectic patients when compared with non-cachectic patients. Equally there was a similar (but not significant) increase in phospho-SMAD3 associated with >5% weight loss. It is not known whether such increased protein levels indicate increased pathway activity independent of any alteration in the ratio of phospho-SMAD3/SMAD3.

It is important to appreciate that the majority of patients in Chapter 4 will have had some degree of age-related sarcopenia, that this will necessarily co-exist with any cancer-specific loss of skeletal muscle mass and that the diagnostic criteria used in the present study will not necessarily separate one from the other. The study reported in Chapter 4

was not longitudinal and it was therefore not possible to document active muscle loss. It is also important to recognise that when patients were divided into different diagnostic categories the sample size in individual categories may have limited the ability to detect a statistical difference or not. This was an exploratory study and provides the basis for a larger study with adequate statistical power for definitive analysis. However, it has demonstrated when the diagnostic criteria for cachexia include both a measure of low muscularity and weight loss, muscle fibre size, protein content and RNA/DNA content are all reduced. Such consistent findings were not observed when cachexia was diagnosed based on weight-loss or low muscularity alone. Whereas fibre type is not targeted selectively, muscle fibre size, biochemical composition and pathway phenotype can vary according to whether the criteria for cachexia include both a measure of low muscularity and weight loss. Such findings suggest that current diagnostic criteria identify groups of patients with different skeletal muscle phenotypes. Identification of a more homogeneous patient cohort for musculo-centric intervention trials may require use of such combined criteria. The findings in Chapter 4 lead on to accurate phenotyping of patients suffering from cancer cachexia and provides novel insights into genetic targets potentially associated with the condition.

7.3 Genetic basis of inter individual susceptibility to cancer cachexia: selection of potential candidate gene polymorphisms for association studies

Following the successful identification of the SNP rs6136 in the SELP gene, a contemporary review of the literature was needed as a greater understanding of the processes involved in cancer cachexia are now available. To enable further candidate gene selection studies with larger cohorts, new targets were identified to maximise the potential for novel associations. To accommodate the evolving phenotype definitions and the current state of the understanding of cachexia, a search was undertaken for candidate

genes or pathways related to the biology of muscle, inflammation, adipose tissue, obesity, diabetes and molecular mechanisms of cancer in general, as well as factors affecting survival and prediction of outcomes following treatments. In view of the increased understanding of the biology of cancer cachexia, the search terms were amended to reflect the evolving biology and classification of cancer cachexia. Such an approach would likely strengthen the premise for hypothesis driven association studies for polygenic diseases/traits, vis-à-vis the potential to identify variants with higher effect size and hence the heritable component of the cachexia risk in individuals.

In Chapter 5 a total of 154 genes associated with cancer cachexia were identified and explored for functional polymorphisms. Of these 154 genes, 119 had a combined total of 281 polymorphisms with functional and/or clinical significance in terms of cachexia. Of these, 80 polymorphisms (in 51 genes) were replicated in more than one study with 24 polymorphisms found to influence two or more hallmarks of cachexia (i.e. inflammation, loss of fat mass and/or lean mass and reduced survival). These 24 polymorphisms are likely to be the most promising candidates in terms of susceptibility biomarkers for cancer cachexia. Since this updated systematic review, one paper has published an association with two SNPs which feature in the review, one being rs6136, further vindicating this candidate gene approach (206). The understanding of cancer cachexia mechanisms is vast as discussed in Chapter 2, by understanding these processes a robust candidate gene analysis can be performed s which makes detection of on meaningful SNPs a likely outcome. Chapter 5 provides a comprehensive framework for the detection of new SNPs in the newly discovered genes regulating the biological processes involved in cancer cachexia since the last one.

Biotechnology is progressing at an unprecedented rate and genome wide association studies (GWAS) are common place. Conducting a GWAS study for cancer cachexia would seem to be the next logical step as they provide a relatively unbiased examination of the entire genome for common risk variants. However in doing so there is potential to dampen the signal from true risk variants with statistical noise from the vast numbers of markers that are not associated with disease. To determine true significance, researchers need a much larger cohort of patients and have to set extremely high significance values. By achieving high significance values the problem of false positives is reduced, but it also

means that any true disease markers with small effects are lost in the background noise (788).

7.4 Genetic signatures associated with susceptibility to cancer cachexia vary according to weight-loss or low muscle mass phenotype

In the study reported in Chapter 6 1276 patients were studied in order to (i) replicate SELP rs6136 and other genes previously associated with WL (ii) explore associations with 92 predefined new candidate single nucleotide polymorphisms (SNPs) and (iii) explore our panel of candidate SNPs (i and ii) for association with CT-defined low muscularity (LM) +/- WL. Also explored was whether the transcription in muscle (n=134 cancer patients) of identified genes was altered according to cachexia phenotype.

7.4.1 Weight loss phenotype: Stage 2 analysis of previously identified SNPs in newly recruited cohort (n=545)

One of the major criticisms from the previous study reported from our group (207) that needed to be addressed was the relatively small size of the validation cohort (n=101). The study reported in Chapter 6 recruited 545 new patients to perform and validate the candidate gene analysis and found two SNPs were validated in this new, larger validation cohort, one being rs6136 found on chromosome 1 in the SELP gene had a significant association with all levels of WL. The SELP gene encodes for p-selectin, a molecule which functions as a cell adhesion molecule (CAM) in response to inflammation on the surfaces of activated endothelial cells. The second SNP (rs4149570), found in the TNFRSF1A gene on chromosome 12 was found to have a significant association with >15% WL. This gene encodes for one of the major receptors for tumour necrosis factor-α. The downstream effects of this receptor are activation of the transcription factor NF-κB to mediate apoptosis, and thus it functions as a regulator of inflammation. These findings confirm rs6136 as the leading replicated SNP to associate with WL.

7.4.2 Weight loss phenotype: Stage 1 analysis with new candidate SNPs in combined cohort (n=1276)

A key issue of any gene association study is the power to detect associations with the rarest phenotype. The study reported in Chapter 6 examined 92 new SNPs in a stage 1 analysis that included 1276 cancer patients, the largest sample size to date in an association study for cancer cachexia. In total, 12 SNPs had significant associations with various cachexia phenotypes based on increasing severity of WL. Six of these SNPs are associated with muscle metabolism in five genes (IGF1, CPN1, FOXO1, FOXO3, and ACVR2B), three are associated with adipose tissue metabolism in two genes (LEPR and TOMM40 (APOE on the reverse strand)), two with corticosteroid signalling in one gene (IFT172 (GCKR on the reverse strand)) and one with the immune response in one gene (TLR4). Two polymorphisms (rs1935949 and rs4946935) in the gene encoding for FOXO3 were consistently associated with WL of increasing severity. On the basis that WL is a continuum in the cachectic process, the observation that both SELP and FOXO3 associate with all degrees of WL suggests that these genetic signatures may be of particular significance. All of these new targets in this stage one analysis represent new SNPs from various different domains discovered by the updated systematic review. This gives potential new insight into the recent mechanisms associated with cancer cachexia.

7.4.3 Low muscularity phenotype: Stage 1 analysis with all candidate SNPs in combined cohort with CT scans (n=751)

One of the main difficulties in research involving cachexia is the lack of a specific phenotype. Chapter 3 has reported that CT scans can be used to derive an index of muscle mass to classify patients with low muscularity or not using established cut-offs for their skeletal muscle index. Moreover the latter can be related to a change in muscle fibre diameter indicating active muscle atrophy associated with cachexia. The study reported in Chapter 6 also investigated the potential relationship between patients' genotype and the propensity to low muscularity in cancer cachexia. In total, 5 SNPs were associated

significantly with the cachexia phenotype based on levels of muscularity: (i) rs4291 in chromosome 17 in the angiotensin converting enzyme (ACE) gene; this gene has been associated with muscle function and metabolism; (ii) rs10636 in chromosome 16 in the metallothionein 2a gene; this gene has been shown to be involved in zinc dyshomeostasis which may contribute to cancer cachexia; (iii) rs1190584 in chromosome 14 in the WDR20 gene; this gene encodes a WD repeat-containing protein that functions to preserve and regulate the activity of the USP12-UAF1 deubiquitinating enzyme complex; (iv) rs3856806 in the peroxisome proliferator-activated receptor gamma (PPARG) gene in chromosome 3 which has been demonstrated to be involved in fatty acid and glucose metabolism; and (v) rs3745012 in chromosome 18 in the lipin 2 (LPIN2) gene; this gene represents a candidate gene for human lipodystrophy, characterised by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance.

7.4.4 Low muscularity plus weight loss phenotype: Stage 1 analysis with all candidate SNPs in combined cohort (n=1276)

In the study reported in Chapter 6, when patients were classified as cachectic according to both WL and LM, four SNPs were identified. Two of these SNPs are associated with muscle metabolism in two genes (ACVR2B and ACE), one with fat metabolism in one gene (LEPR), and one with cytokine production in one gene (TNF). The observation that most of the identified genes are associated with muscle and adipose tissue metabolism highlights the importance of these processes in the progression of cancer cachexia. Skeletal muscle wasting is a signature event in cancer cachexia and can be associated with increased fatty infiltration (763), therefore mechanisms effecting the functions of these genes may be of particular significance. It would be attractive to assign specific functional significance to some of the genetic signatures so identified. For example ACVR2B decoy receptors have been shown to abrogate muscle loss and prolong survival in murine models of cancer cachexia (145).

7.4.5 Combining genes with functional similarity according to gene ontology

Candidate genes (and the SNPs in the corresponding gene regions) were grouped based on known functional similarity according to gene ontology and the association between grouped SNPs and cachexia was analysed using subjects in the main study cohort. Gene group analysis provides one way of summarising the evidence between cachexia traits and multiple genetic variants across groups of genes that share functional similarity.

SNPs in groups of genes involved in appetite regulation, cell adhesion, cell membrane structure and function, and signal transduction were associated with the phenotype WL >10%. Only SNPs in the group of genes involved in cell adhesion were significant with increasing severity of WL. SNPs in groups of genes involved in lipid metabolism, appetite regulation, signal transduction and glucocorticoid signalling were associated with the phenotype LM and WL >2%. No SNPs in groups of genes were found to be significant with all other phenotypes.

There is some evidence that negative regulators of appetite are elevated in cancer cachexia (13, 688, 690, 691). Lipid metabolism was significantly associated with the cachexia trait low muscle mass and WL >2% (p=0.0138). Fatty infiltration resulting in reduced muscle quality (myosteatosis) has been associated with cancer cachexia and reduced survival (396, 763). The glucocorticoid signalling pathway was also found to be associated with the cachexia trait LM and WL >2% (p=0.0337). Glucocorticoids and associated signalling pathways have been implicated in accelerating protein degradation in muscle, which results in loss of lean body mass in cachexia (100). Glucocorticoids are thought to act through a permissive effect on the upregulation of messenger RNA and the subsequent synthesis of components of the ubiquitin– proteasome system in muscle. Glucocorticoids may also inhibit protein synthesis and promote gluconeogenesis, and suppress glucose and amino acid muscle uptake by inhibiting cellular transporters (766).

7.4.6 Differential gene expression signatures in human muscle and their association with the phenotypes of cachexia

Transcriptomic analysis for gene transcript level for the genes that showed significant associations with any of the cachexia phenotypes was undertaken. Expression of ACVR2B, FOXO1 and 3, LEPR, PPARG, TLR4, and TOMM40 transcripts was significantly associated with different levels of SMI or WL ($P<0.05$). Specifically, these were all negatively correlated with muscularity. FOXO1 and 3 and TOMM40 were the only genes significantly correlated with WL; these were correlated negatively with WL. It has previously been shown that the presence of cancer cachexia is associated with the transcriptional regulation of about 1800 genes in human skeletal muscle (177). It was therefore of interest to see if the transcript level of the genes associated with cachexia in Chapter 6 were altered in a separate cohort of cancer patients with or without cachexia. Indeed, there was a strong concordance between a proportion of the selected genes and either the level of WL or muscularity. FOXO1 and FOXO3 are good examples: SNPs in both genes were identified to associate with the WL phenotype and transcript levels of both showed a correlation with WL. These transcription factors are key in the pro-inflammatory driven up-regulation of the ubiquitin-proteasome pathway, but also act as a negative regulator of the anabolic Akt-mTOR pathway (95). Inhibition of FOXO transcriptional activity prevents muscle fibre atrophy during cancer cachexia and induces hypertrophy (95). It is therefore plausible that variants of the FOXO genes are key determinants of the muscle loss component of cachexia syndrome.

7.4.7 Limitations

In the study reported in Chapter 4 it is important to appreciate that the majority of patients in the present series will have had some degree of age-related sarcopenia, that this will necessarily co-exist with any cancer specific loss of skeletal muscle mass and that the diagnostic criteria used in the present study will not necessarily separate one from the other. The study reported in Chapter 4 was not longitudinal and it was therefore not possible to document active muscle loss. It is also important to recognise that when patients were divided into different diagnostic categories the sample size in individual

categories may have limited the ability to detect a statistical difference or not. This was an exploratory study and provides the basis for a larger study with adequate statistical power for definitive analysis.

In the study reported in Chapter 5 eighty eight newly identified genes with a potential role in cancer cachexia were included since the last review, however the main limitation to identifying new SNPs in these genes was the lack of studies to date into functional polymorphisms within them. Undertaking a genome wide association study (GWAS) would be one way to overcome this potential limitation and in the future would be preferable to repeating candidate gene selection studies. However, without an adequate sample size and highly accurate phenotyping, coupled with lack of government funding to complete the project, candidate gene association studies will continue to provide novel insights in to the genetic of cancer cachexia.

The analyses in the study reported in Chapter 6 was not of a genome-wide nature and therefore genetic variants with much greater functional significance may have not have been discovered. Equally, the true functional significance of any individual SNP for cancer patients is mostly unknown. It may, therefore, be better to consider the genetic associations identified in the present analysis as genetic signatures or biomarkers associated with the cachexia syndrome. Interestingly, 17 of the 19 SNPs reported as showing significant associations with the cachexia phenotypes are in intronic, 3', or 5' UTRs. The purpose was to explore the trends and probe into the potential functional impact of the loci as SNPs in this study are potentially proxy to the more causal variants (not yet captured in the region) which may also have an influence on gene expression; as such the probe position in the expression array and the SNP position are not the same. Extrapolation to a SNP under investigation being an expression quantitative loci (eQTL) is premature. The SNP identified may in some cases also affect gene expression signatures not addressed herein (as in cis- and trans acting eQTLs). The correlation pattern (albeit, low to modest) observed is still encouraging since the trends reported here for a SNP loci and gene expression are within the scope of known cachexia literature. Further replication of the present findings and fine mapping of the loci may offer insights. Independent of the functional significance of the polymorphisms, the SNP signatures identified herein serve as heritable determinants for cancer cachexia; the

polygenic nature of the phenotype warrants identification of more susceptibility variants to enable stratification of patients for cancer cachexia risk.

Equally, for those genes for which no strong relationship was found between gene expression and patient characteristics it is important to consider that these may not be transcriptionally regulated. For the various humoral mediators (ACE, TNF, IGF1), it may be that the concentration in blood is more important rather than local expression in tissues since tissue-specific expression may be transient for some genes but the activation of the signal transduction cascade could be the largest prevailing effect. For instance local expression of the ligand may be a small part of a bigger picture where several tissue sources of ligand, a variety of receptors, soluble receptors and binding proteins may play a role in the actual response (e.g., TNF and IGF-1).

The study reported in Chapter 6 is part of a series of studies undertaken by our international consortium which over time has expanded DNA biobanking and cachexia phenotyping from 775 to 1276 patients. While these represent the largest candidate gene association studies done to date in relation to cancer cachexia, they have the general limitation of the candidate gene approach. The assembly of much larger data sets and samples in order to conduct genome wide association studies is awaited.

It is important to establish that the prevalence of LM in the present cancer cohort is in excess of that which would be observed in the normal age-matched population. The prevalence of LM/sarcopenia in age-matched subjects living in the community varies according to the precise definition and methodology used, but is reported to be between 1-29%. The prevalence of LM that was observed in the cancer cohort in Chapter 6 was 44% for males and 52% for females. Thus, the gene associations established with LM represent associations with a level of muscularity at least partly independent of age, sex or stature.

Selection of the genes and SNPs may be biased towards genes with known potential to influence on WL. Therefore genes used for other processes associated with cachexia such as muscle and lipid regulation are under-represented and potential associations may have been missed in these key regulatory pathways. As many of these genes have been recently discovered there is paucity of functional significance. As far as the muscle and lipid regulation phenotypes are concerned, the present study begins a journey into

defining phenotypes based on these novel measurements. Other independent studies need to be conducted to address the muscle loss phenotype and its associated genes and pathways. It is also important to recognise that the understanding of the biology of cachexia is evolving rapidly and that factors such as GDF-15 (MIC-1) that might now be considered as potentially important (789), were not included in the present study.

The candidate gene SNP analysis offers the advantage that it is hypothesis driven and the identified associations are easily explained due to compelling biological rationale. However, the limitations of this approach are that the role of hitherto unexplored genes and pathways that otherwise contribute to the trait under investigation are missed. Issues surrounding phenotype complexity are addressed in part in this study and conducting a genome wide association study (GWAS) using high density of markers on the genome would help relate the overlap of SNPs/pathways to the phenotypes of interest. The consensus definitions for phenotypes may evolve in an iterative manner from the cumulative wisdom from candidate SNPs, GWAS and the current definitions available for cachexia.

7.5 Future work and direction

Chapter 2 looked at the vast amount of research conducted on cachectic muscle in both animal models and clinical studies. The heterogeneity between morphology, mediators, and mechanisms of animal models and human cancer cachexia is considerable. There are some comparisons that can be drawn from the two but it must be realised that these may represent distinct disease processes. The relatively acute wasting seen in young, metabolically active animal models may have little bearing on the chronic disease patho - physiology seen in aged cancer patients with chronic wasting. Progress in treating the human form of cancer cachexia can only move forwards through carefully designed large randomised controlled clinical trials of specific therapies with validated biomarkers of relevance to underlying mechanisms. This thesis sought to identify SNPs as biomarkers for such trials. From the results in Chapter 6 it can be assumed that rs6136 in the SELP gene is a novel biomarker in the development of cancer cachexia as defined by increasing weight loss. It could also be assumed that rs2268757 in the ACVR2B gene

has the potential to be a novel biomarker in the development of cancer cachexia as defined as increasing muscle and weight loss. The observation that many of the significant SNPs in each phenotype were from genes directly associated with the pathophysiology of that same phenotype only confirms the need for accurate phenotyping which was demonstrated in muscle in Chapter 4. The latter chapter demonstrated that a reduction in fibre diameter was seen when an element of muscularity was introduced to phenotyping based on weight loss alone. Further work is needed to validate these findings in a larger cohort as this was an exploratory study. Nonetheless Chapter 4 validates the definition proposed by the international consensus that any weight loss (>2%) in the presence of low muscularity will have biological significance (11). Of the new significant SNPs identified in Chapter 6, the SCAN database highlights some of the genes whose expression may be altered. It would be of interest to look at the transcript levels of some of the potentially more influential genes in terms of cancer cachexia in the current cohort. Clearly a GWAS study is needed in the future to take forward the genetics of cancer cachexia, however for the reasons outlined in the present Chapter it needs to be carefully planned with the appropriate large number of patients.

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Appendices

Appendix A

04 January 2007

Professor KCH Fearon
Professor of Surgical Oncology
Clinical and Surgical Sciences (Surgery)
The University of Edinburgh
Royal Infirmary of Edinburgh
51 Little France Crescent, Edinburgh, EH16 4SA

Dear Professor Fearon

Full title of study: **The Identification of Early Biochemical and Clinical Markers of Cancer-Associated Wasting**
REC reference number: **06/S1103/75**

Thank you for your communication of 22 December 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered on behalf of the Committee by the Chair, Dr Christine West.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of approval
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	AB/95114/1	08 December 2006
Investigator CV		
Protocol	2.1	03 November 2006
Covering Letter	with original submission	08 December 2006
Letter from Sponsor		04 October 2006
Questionnaire: Package of Questionnaires	1.1	03 November 2006
GP/Consultant Information Sheets	2.1	03 November 2006
Participant Information Sheet: GP Info Sheet for Controls	1.1	03 November 2006
Participant Information Sheet: for controls	2.1	03 November 2006
Participant Information Sheet	2.1	03 November 2006
Participant Consent Form: for controls	2.2	22 December 2006
Participant Consent Form	2.2	22 December 2006
Letter from Dr Heather Cubie - Management approval		17 November 2006
Letter from Cancer Research UK		29 March 2006
Letter from LREC01		08 November 2006

Research governance approval
The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC Reference Number	**06/S1103/75**	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Chair
Lothian Local Research Ethics Committee 03
Email: elizabeth.harden@lhb.scot.nhs.uk

Enclosure: *Standard approval conditions*

Copy to: Ms Marise Brown
University of Edinburgh
Clinical Trials & Research Governance Manager
College of Medicine & Veterinary Medicine, Office E1.06
Queen's Medical Research Institute
47 Little France Crescent, Edinburgh, EH16 4TJ

R&D Department for NHS Lothian

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15 July 2008

Prof KCH Fearon
Professor of Surgical Oncology
Clinical and Surgical Sciences
University of Edinburgh
ROYAL INFIRMARY OF EDINBURGH
51 Little France Crescent
Edinburgh EH16 4SA

Dear Prof Fearon

Full title of study:	**Identification of genetic markers of cancer cachexia**
REC reference number:	**08/S1103/19**

Thank you for your letter dated 26 May 2008 which arrived in the LREC office on 30 June 2008, responding to the Committee's request for further information on the above research and submitting revised documentation, subject to the conditions specified below.

The further information was recently considered by the chair on behalf of LREC 3.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		28 April 2008
Investigator CV	CI	
Protocol	1	22 February 2008
Covering Letter		24 April 2008
Letter from Sponsor		17 April 2008
GP/Consultant Information Sheets	1.1	07 April 2008
Participant Information Sheet: PIS	2.1	25 June 2008
Participant Consent Form	2.1	25 June 2008
Response to Request for Further Information		26 May 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/S1103/19	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Appendix B

Patient Information Sheet (Genetics)

THE IDENTIFICATION OF GENETIC 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 if there is a genetic basis for 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 if there is any way to predict its development, we would like to see if wasting in cancer is related to the presence or absence of certain genetic markers.

Why have I been chosen?

You have been chosen because you have been diagnosed with oesophageal, gastric or pancreatic cancer

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 a single blood test and have your weight and height measured. This will take place during a routine outpatient clinic appointment with your consultant. No extra appointments are required if you choose to take part in this study. We will also ask your permission for

access to your clinical case notes and any CT scan images that are performed as part of your routine treatment.

- ***Blood test:*** The amount of blood taken is approximately the same as one teaspoonful (15ml), and it will be taken from your arm in the usual fashion.

What do I have to do?

Apart from attendance at the initial appointment, no other responsibilities are required from your participation.

What are the possible disadvantages and risks of taking part?

There are negligible disadvantages and risks of taking part. Minor discomfort may occur during blood sampling.

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 initial appointment, no further appointments are required. However, if any of your blood, or DNA 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. All information that was collected during the time of your participation in the study will be destroyed. Any stored blood or DNA samples that are identified as yours will also be destroyed

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.

Blood and DNA samples collected during the study may be transferred for the purpose of analysis to associated researchers within 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 sample will be immediately analysed by the Department of Biochemistry at the Royal Infirmary of Edinburgh or Western General Hospital. The remainder of the blood sample 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 study research team. DNA will be extracted from the blood sample at the University of Edinburgh. A small portion of the DNA will be sent from the University of Edinburgh to our collaborators at the University of Alberta, Canada, for genetic analysis. The samples that are sent to Canada will already be anonymised so that you will not be identifiable from them.

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?

Yes. There will not be any implications, healthcare or otherwise, from the genetic study. The genetic markers that we aim to test for have not been shown to be linked with or have any association with inheritable risk for any known diseases. Therefore, we will not normally feedback any results 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. 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 the European Commission.

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 the European Commission.

Contact details

You may contact our research team directly by telephoning 0131 242 6520 for further information at any time. Alternatively, you may contact Prof. Stephen Wigmore, Consultant Surgeon in the Department of Surgery, who is acting as an independent advisor – contact 0131 242 3615.

Many thanks for your time.

Patient Information Sheet (Muscle biopsy)

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 with cancer if they would undergo a variety of tests around diagnosis, and 6 months, 12 months and 18 months afterwards. 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, 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 are undergoing treatment for cancer.

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 around the time of diagnosis, and at appointments 6 months, 12 months and 18 months afterwards. The appointments will be timed to coincide with the normal clinic appointments where possible. 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 is approximately the same as one teaspoonful (5ml), 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.

- ***An MRI scan of your legs:*** This test will be carried out in the Department of Radiology. It involves you lying flat within the scanner and will take approximately 20 minutes to perform. 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. 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 afterwards.

Apart from the above-described tests, the only other test will be to take a muscle biopsy:

- ***A muscle biopsy:*** We plan to biopsy the muscle on the front of your thigh. The amount of muscle removed is small and is taken via a tiny incision (a few millimeters long) through which a needle is introduced. The procedure is performed under local anaesthetic so you will not feel any pain. This incision will be closed by paper stitches and will leave a small mark which will fade.

If at one of the appointments dated 6 months, 12 months and 18 months after the initial assessment you are noticed to have lost a significant amount of weight (e.g. more than 15% of your normal weight), we would ask that you give us permission for a further thigh muscle biopsy at that time. This will again be performed under a local anaesthetic injection. 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 initial appointment and at 6, 12 and 18 months afterwards, 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. This biopsy will be performed under a local anaesthetic injection. The anesthetic agent may sting for a few seconds as it is being administered. Also, approximately 1 in 200 people suffer with bruising following the procedure. 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 at 18 months, no further appointments are required. However, if any of your blood, muscle 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 and blood 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 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 the research team 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.

Appendix C

Patient Consent Form (Genetics)

CONSENT FORM

THE IDENTIFICATION OF GENETIC MARKERS OF CANCER- ASSOCIATED WASTING

1. I agree to take part in the above-titled study. ☐

2. I agree for my blood to be taken for the purpose of DNA analysis. ☐

3. I confirm that I have read and understand the information sheet dated June 2008 (version 2.1) for the above study. I have had the opportunity to consider the information and ask questions, and I have had these answered satisfactorily. ☐

4. 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. ☐

5. 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. ☐

6. I agree to my GP being informed of my participation in the study. ☐

7. 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). ☐

Name of Patient	Date	Signature
Researcher	Date	Signature

Patient Consent Form (Muscle biopsy)

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

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REVIEW ARTICLE

Genetic basis of interindividual susceptibility to cancer cachexia: selection of potential candidate gene polymorphisms for association studies

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Abstract

Cancer cachexia is a complex and multifactorial disease. Evolving definitions highlight the fact that a diverse range of biological processes contribute to cancer cachexia. Part of the variation in who will and who will not develop cancer cachexia may be genetically determined. As new definitions, classifications and biological targets continue to evolve, there is a need for reappraisal of the literature for future candidate association studies. This review summarizes genes identified or implicated as well as putative candidate genes contributing to cachexia, identified through diverse technology platforms and model systems to further guide association studies. A systematic search covering 1986–2012 was performed for potential candidate genes / genetic polymorphisms relating to cancer cachexia. All candidate genes were reviewed for functional polymorphisms or clinically significant polymorphisms associated with cachexia using the OMIM and GeneRIF databases. Pathway analysis software was used to reveal possible network associations between genes. Functionality of SNPs/genes was explored based on published literature, algorithms for detecting putative deleterious SNPs and interrogating the database for expression of quantitative trait loci (eQTLs). A total of 154 genes associated with cancer cachexia were identified and explored for functional polymorphisms. Of these 154 genes, 119 had a combined total of 281 polymorphisms with functional and/or clinical significance in terms of cachexia associated with them. Of these, 80 polymorphisms (in 51 genes) were replicated in more than one study with 24 polymorphisms found to influence two or more hallmarks of cachexia (i.e., inflammation, loss of fat mass and/or lean mass and reduced survival). Selection of candidate genes and polymorphisms is a key element of multigene study design. The present study provides a contemporary basis to select genes and/or polymorphisms for further association studies in cancer cachexia, and to develop their potential as susceptibility biomarkers of cachexia.

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Introduction

Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life and duration of survival. Cancer cachexia has recently been defined as a multifactorial syndrome characterized 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 (Fearon *et al.* 2011). Depending on the tumour type, weight loss occurs in 30–80% of cancer patients and is severe (with loss of >10% of the initial body weight) in 15% (Dewys *et al.* 1980). The degree of cancer cachexia is variable depending on the phenotype and genotype of both patient and tumour. It is likely, there may be cachexia prone genotypes as well as cachexia resistant genotypes. A recent longitudinal study of patients with a variety of cancers has demonstrated that some will remain

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stable, lose or gain skeletal muscle or adipose tissue (Prado *et al.* 2013), further strengthening the concept of a genetic predisposition to wasting in the presence of cancer.

Pathophysiology of cancer cachexia is characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism (Fearon *et al.* 2011). Skeletal muscle loss appears to be the most significant event in cachexia leading to poor treatment outcomes (Tan *et al.* 2009; Fearon *et al.* 2011). While malnutrition is reversible with nutrient intake, cachexia is not completely reversible by this approach. Indeed, cachectic patients usually present with progressive weight loss along with body composition alterations and disturbed homeostasis of many body systems, particularly of fat tissue and muscle (Tisdale 2002; von Haehling *et al.* 2009).

Single nucleotide polymorphisms (SNPs) are the most common type of heritable and evolutionarily stable genetic variations in the population (Brookes 1999); other genetic polymorphisms include copy number aberrations, insertion, deletions and tandem repeats. SNPs may exert differing effects on genes leading to an aberrant gene product. Polymorphisms in promoter regions potentially contribute to differential gene expression, presumably affecting the binding of transcription factors to DNA. Sequence variation in the 5′ untranslated region (UTR) could disrupt mRNA translation; mutations in the 3′ UTR could affect mRNA through posttranscriptional mechanisms such as splicing, maturation, stability and export. Polymorphisms in intronic regions may result in *cis* regulation or *trans* regulation of genes, unmask cryptic splice sites or promoters leading to alternative transcripts. Synonymous and nonsynonymous SNPs in exons could alter protein function or activity and may introduce codon bias contributing to the relative abundance of the proteins, respectively. Finally, nonsense mutations cause a stop altogether in the translation of mRNA (Wjst 2004). Genomic distribution of SNPs is not homogenous, SNPs usually occur in noncoding regions more frequently than in coding regions (Barreiro *et al.* 2008). It has been estimated that 10% of all SNPs in the genome are functional, thereby having the potential of altering some biological process.

The case to support a genetic predisposition to cachexia is strengthened from the known genetic contribution to the activity of a variety of key mechanisms that underlie the cachexia syndrome. In a previous systematic review on the identification of possible genetic polymorphisms involved in cancer cachexia, a total of 184 polymorphisms with functional or clinical relevance to cancer cachexia were identified in 92 candidate genes (Tan *et al.* 2011). Following this approach here, we were able to identify 23 significant SNPs associated with cachexia based on definitions of weight loss and systemic inflammation (measured with C-reactive protein (CRP)) and validated a SNP in the *SELP* gene encoding for p-selectin in an independent cohort. P-selectin binds to leucocytes and in certain inflammatory conditions, the plasma concentration of soluble p-selectin is highly elevated (Dunlop *et al.* 1992). By incorporating a definition based on systemic inflammation, we identified a SNP involved in the innate immune response. To date most of the studies on identification of SNPs involved in cancer cachexia has led to the discovery of other SNPs involved in the innate immune system, mainly in the interleukin family of cytokines (Jatoi *et al.* 2007, 2010; Knoll *et al.* 2008; Zhang *et al.* 2008; Deans *et al.* 2009; Bo *et al.* 2010; Rausch *et al.* 2010; Sun *et al.* 2010a, b) (table 1). SNPs in other biological processes have also been studied for their association with cancer cachexia (Solheim 2011, 2012; Punzi *et al*. 2012) (table 1). By altering the phenotype for a degree of skeletal muscle quantification, it may be possible to identify SNPs associated with muscle tissue biology.

A contemporary review of the literature is now needed as a greater understanding of the processes involved in cancer cachexia are increasingly sought. To enable further candidate gene selection studies with larger cohorts, new targets need to be identified to maximise the potential for associations. An increase in earlier sample size will increase the power of further studies to identify novel SNPs. To accommodate the evolving phenotype definitions and the current state of the understanding of cachexia, we searched for candidate genes or pathways related to the biology of muscle, inflammation, adipose tissue, obesity, diabetes and molecular mechanisms of cancer in general, as well as factors affecting survival and prediction of outcomes following treatments. There is an express need to restrategize the selection of candidate genes to drive association studies since 'omics' approaches uncovered the hitherto unexplored biological pathways in the last five years in the realm of cancer and genetic predisposition markers for a host of diseases and traits. In view of the increased understanding of the biology of cancer cachexia, we have amended the search terms used as both biology and classification of cancer cachexia continues to evolve. We reasoned that insights into functional significance of the candidate gene SNPs will help explore putative causality of the SNPs. These approaches would likely strengthen the premise for hypothesis driven phenotype–genotype association studies for polygenic diseases/traits, *vis-à-vis* potential to identify variants with higher effect size and hence heritable component of the cachexia risk in individuals. Functionality of SNPs/genes was explored based on published literature, algorithms for detecting putative deleterious SNPs and databases for expression quantitative trait loci (eQTLs) (Hunter and Crawford 2008; Schadt *et al.* 2008; Fehrmann *et al.* 2011; Hao *et al.* 2012; He *et al.* 2013). Interindividual variations in susceptibility to cancer induced cachexia and the heritable component of the genome when fully delineated would help identify possible interventions for those at risk well before the onset of clinical symptoms.

Methods

The US National Library of Medicine database, Medline; the Excerpta Medica database, Embase; Cochrane Central Register of Controlled Trials, Central; and the database of the

Table 1. Published associations of genetic variants in cancer cachexia.

Reference	SNP	Gene	Patients included	Phenotype
Solheim (2012)	No associations	N/A	Patients 1853, with cancer at different sites, stages and with different performance status	EORTC QLQ-C30 questionnaire, question 13: 'have you lacked appetite'
Punzi (2012)	rs1544410 (*Bsm*I) rs731236 (*Taq*I)	*VDR*	Patients 43, with cancer from various sites and stages	Guidelines for diagnosis of cancer associated cachexia provided by the Italian Association of Medical Oncology
Tan (2012)	rs6136	*SELP*	Patients 775, with upper gastrointestinal cancers (UGI) and pancreatic cancers	Six phenotypes (1) >5% weight loss (2) >10% weight loss (3) >15% weight loss (4 – 6) The above with CRP concentration of >10 mg/l $^{-1}$
Solheim (2011)	No associations	N/A	Patients 1797, with cancer at different sites, stages and with different performance status	• BMI: <20 kgm^{-2} • Karnofsky score: <80 • CRP: <10 mg l^{-1} • Appetite loss: a response of little or greater on EORTC QLQ-C30 item 'have you lacked appetite?' >3 features = severe cachexia 2 or 3 features = mild cachexia <2 = no cachexia
Sun *et al.* (2010a, b)	rs1800896	*IL-10*	Two hundred and twenty-three gastric cancer	
Bo *et al.* (2010)	rs2227306	*IL-8* +781	Gastric cancer	
Jatoi *et al.* (2010)	rs1800629	*TNF*	Patients 471, with non small cell lung cancer	>10% Weight loss
Rausch *et al.* (2010)	rs3024498	*IL-10*	Caucasian lung cancer survivors 1149	Lung cancer symptom scale How much loss appetite are you experiencing?
Deans *et al.* (2009)	rs1800896	*IL-10* (-1082)	Patients 203, with UGI cancers	>10% Weight loss
Knoll *et al.* (2008)	rs2229616 Val103Ile	*MC4R*	Patients 509, with various cancers (including haematological malignancies) at various stages	(1) >10% weight loss (exclusively cancer) (2) >10% weight loss (treatment influenced) (3) >5% weight loss (4) >1% weight loss (cancer specific) or >5% treatment induced (5) No weight loss
Zhang *et al.* (2007)	rs1143634	*IL-1*β (+3954)	Patients 214, with locally advanced gastric cancer	>10% Weight loss
Jatoi *et al.* (2007)	rs1143634	*IL-1*β (+3954)	Patients 44, with metastatic gastric and gastrooesophagal cancer	Phenotype is greater improvement in weight registered once in every three weeks during chemotherapy

BMI, body mass index.

Cumulative Index to Nursing and Allied Health Literature, Cinahl, were searched through the National Library of Health website, the Cochrane library, Pubmed (free citation database of Medline) and Grey literature online.

The first aim was to identify articles with new potential candidate genes involved in the development of cancer cachexia using the keyword search 'genes/genetics and inflammation or cancer or cachexia or weight loss or body composition or survival or muscle or adipose to provide an update on the existing database. Following the initial retrieval of possible new candidate genes, selected genes were entered into a pathway functional analysis software (ingenuity pathways analysis (IPA), ingenuity systems, Redwood, USA) to further identify related genes.

The second and third aims were to find genetic polymorphisms with known functional or clinical significance in each of these new and existing genes using the keyword search (gene of interest) and polymorphism(s). All identified candidate genes were reviewed for functional polymorphisms or clinically significant polymorphisms in terms of cachexia using OMIM, dbSNP and GeneRIF databases. The search was limited to reports within English language and a manual search of reference lists and conference proceedings followed with all cross references screened.

Study selection

Published papers and or abstracts were screened after removing duplicates. Inclusion criteria were set as any study either involving genes in the selected domains or any study showing functional SNPs influencing these genes. Reasons for exclusion included studies of irrelevance to cancer cachexia, studies with no genes or functional polymorphisms identified, and complex polygenic phenotypes analysing random SNPs for association. The remaining papers were reviewed in full and only those involving genes in the development of cancer cachexia or any study showing functional SNPs influencing these genes, were included in the final analysis.

In accordance with the previous review, candidate genes were grouped based on the role their product is postulated to have in the development of cancer cachexia (Tan *et al.* 2011), these being the domains of:

(i) Inflammation:
(a) innate immune receptors and mediators of the immune response; (b) cytokines; (c) cytokine receptors and related binding proteins; (d) acute phase protein reactants.
(ii) Central homeostasis
(a) energy production; (b) insulin-like growth factors and related proteins; (c) corticosteroid signalling proteins.
(iii) Muscle:
(a) muscle function and structure; (b) muscle synthesis; (c) muscle proteolysis and degradation.
(iv) Adipose tissue:
(a) adipogenesis; (b) lipid turnover and transport; (c) adipokines and adipokine receptors.
(v) Appetite.
(vi) Others.

The updated summary tables of polymorphisms are presented according to each category with 'easy to see' boxes that denote whether a polymorphism has any effect on inflammation, weight/body composition (i.e. lean mass / fat mass) and cancer survival. Also added is information on ancestral allele, SNP allele, type of SNP, polymorphism reference number (rs number), and mean allele frequency (MAF) based on a population with European ancestry derived from the HapMap or dbSNP databases.

Pathway analysis

Multiple genes within a single pathway are likely to influence the development of cancer cachexia. To provide a more comprehensive assessment in terms of pathway involvement in cancer cachexia, we performed pathway-based analyses using the IPA software. Focus genes were defined as functional SNPs which had been validated in at least one study and these were entered into the IPA analysis tool. The IPA software was used to measure associations of these genes with other molecules, their network interactions, and biological functions stored in its knowledge base. Our focus genes served as seeds for the IPA algorithm, which recognizes functional networks by identifying interconnected molecules, including molecules not among the focus genes from the IPA knowledge base. The software illustrates networks graphically and calculates a score for each network, which represents the approximate 'fit' between the eligible focus molecules and each network. The network score is based on the hypergeometric distribution and is reported as the −log (Fisher's exact test result).

Putative functions

To identify the likely effect of individual SNPs on nearby genes the sorting intolerant from tolerant (SIFT; http://sift.bii.a-star.edu.sg/) program was used for nonsynonymous SNPs (Ng and Henikoff 2003), SIFT is a program that predicts whether a nonsynonymous SNP leading to an amino acid substitution affects protein function or not (Ng and Henikoff 2003). To predict whether an amino acid substitution in a protein will affect protein function, SIFT considers the position at which the change occurred and the type of amino acid change. Given a protein sequence, SIFT chooses related evolutionarily conserved proteins and amino acid residues likely critical for structure or function and obtains an alignment of these proteins with the query. Based on the amino acids appearing at each position in the alignment, SIFT calculates the probability that an amino acid at a position is tolerated conditional on the most frequent amino acid being tolerated. If this normalized value is less than a cut-off, the substitution is predicted to be deleterious (Ng and Henikoff 2001). Many of the amino acid substitutions are well tolerated since the SNPs tend to capture subtle effects, and the ones with drastically altered functions are not to the survival advantage of the species, hence are eliminated during the course of evolution. A limitation of SIFT program is that only nonsynonymous substitutions are interrogated and those in the regulatory and intronic regions (>95% of the genome) are not considered.

The SNP and copy number annotation (SCAN) database (http://www.scandb.org/newinterface/about.html) is a large-scale database providing a web interface for easy search with a gene name or an input of SNP ID (rs#) (Gamazon *et al.* 2010). This database can use SNPs from 5′ or 3′ and intronic SNPs and their potential influence on the gene expression for nearby genes; gene expression if regulated on the same chromosome and within 100 Mb distance from the SNP could be categorized as *cis* regulatory SNPs, if not they are called *trans* SNPs. The SNPs are classified according to their effects on expression levels, i.e. eQTLs using scan database. Gene expression data and polymorphism data from HapMap population-derived lymphoblastoid cell lines (Caucasian, Yoruba and Han Chinese/Japanese) are used to interrogate the *cis* effects and *trans* effects from SNPs/genes. We have therefore summarized our findings for the selected SNPs for possible functional significance using this approach as well. Generalizability of observations

from genotype to expression from lymphoblastoid cell lines is not the same as correlations made with tissue specific expression, and therefore, one should exercise caution in the interpretations of findings. However, several published studies attest to the utility of this approach to extrapolating genotype–phenotype correlations to be valid even to specific tissues, albeit for select candidate SNPs.

Results

A total of 281 polymorphisms in 154 genes were identified and explored for relevance to cancer cachexia (see tables 1–15 in electronic supplementary material at http://www/ias.ac.in/jgenet/). Of these genes, 88 were newly identified and 66 were from the previous review by our group (Tan *et al*. 2011). The 281 functional polymorphisms with or without clinical significance to cancer cachexia (i.e. inflammation, loss of fat mass and/or lean mass and reduced survival) were found in 119 genes with no SNPs of interest found in the remaining 35 genes. A pathway-based analysis for relevance to cachexia and SNPs selected from these pathways are summarized (table 3; figures 1 & 2).

Inflammation

Inflammation is widely accepted to play a dominant role in the host's response to cancer and mediators regulating skeletal muscle atrophy in cachexia are thought to derive from immune or tumour cells, or the targeted tissues undergoing wasting (both adipose tissue and skeletal muscle) (Stewart *et al.* 2006). Cancer cells rely on production of proinflammatory mediators for growth, protection from apoptosis, and promotion of angiogenesis/metastasis. The tumour may consequently initiate a cytokine cascade that has multiple, direct, and distant effects including the initiation of skeletal muscle protein degradation. Host immunological response to proinflammatory and antiinflammatory cytokines dictates to what degree the metabolic rates are altered. In experimental models, proinflammatory cytokines lead to an acute phase response and tissue catabolism (Argiles *et al.* 2003).

Innate immune receptors and mediators of immune response: Electronic supplementary table 1 shows the genes involved in the regulation of the innate immune response and how they may generate or suppress the inflammatory response to influence the rate of cancer cachexia. Variants of these genes which may influence their function are also listed. Since the previous review, focus has spread to include new genes (*AKT2*, *AKT3* and *COX2*) (Bonetto *et al.* 2011; Ol *et al.* 2011) which act via signalling pathways to alter the innate immune response. AKT regulates cellular survival and metabolism by binding and regulating many downstream effectors, e.g. NF-κB and Bcl-2 family proteins (Song *et al.* 2005). IGF-1-induced AKT signalling is important in both the suppression of degradation and induction of protein synthesis (Rommel *et al*. 2001). AKT2 has been shown to induce glucose transport, a mouse model null for AKT2 demonstrated marked growth deficiency and displayed a diabetic phenotype (Garofalo *et al.* 2003); the role of AKT3 is less clear.

Also of interest are variants coding for *COX2*, it is found in low levels in most cells under normal conditions but has been shown to be elevated during periods of inflammation. *COX2* mediates the formation of prostaglandins from arachidonate and may play a role as a major mediator of inflammation and/or a role for prostanoid signalling (Kim *et al.* 2005).

New variants in the genes coding for the toll-like receptor (TLR) family and CAMs have been found. These genes play an instructive role in innate immune responses as well as the subsequent induction of adaptive immune responses. TLRs are involved in triggering intracellular signals, culminating in the activation of nuclear factor (NF)-κB, where it participates in enhancing expression of other immuneregulatory substances (Kawai and Akira 2006), whereas CAMs are known to mediate migration of cells to sites of inflammation.

Cytokines and cytokine receptors: Mediators regulating cancer cachexia are thought to be derived from immune or tumour cells, or the targeted tissues undergoing wasting (both adipose tissue and skeletal muscle). All cytokines play a role in the induction of immune and inflammatory responses. Consequences on cellular function are vast including, proliferation, mediating intracellular tissue cross talk, chemotaxis and killing. Genetic variants of genes encoding proinflammatory and antiinflammatory cytokines are presented in table 2 in electronic supplementary material.

Cytokines bind to their appropriate receptor to initiate a downstream cascade of intracellular signalling. Signalling leads to either potentiation of the signal and to production of similar or other cytokines and their receptors or to a suppression of the signal (Ihle *et al.* 1995). Since the last review, the gene encoding the IL-6R protein has been shown to play an important role in the mediation of IL-6 signalling, and a genetic variant in this gene has been shown to influence the C-reactive protein (CRP) level (Ridker *et al.* 2008). Genetic variants of genes encoding cytokine receptors and related binding proteins are presented in table 3 in electronic supplementary material.

Acute phase protein reactants: An organism responds to the presence of acute infection, tissue injury, trauma or surgery by mounting an acute phase response (APR); this is designed to help limit tissue injury by the increased synthesis of key defence / repair proteins by the liver. However, in certain circumstances, when dietary protein intake is limited and the APR is prolonged or severe, an APR can exacerbate muscle wasting by increasing the demands for certain amino acids to support increased hepatic export protein synthesis. They have also been shown to be predictors of adverse outcomes in cancer patients (Stephens *et al.* 2008). Some acute phase proteins also have roles in modulating immune response such as CRP. CRP genetic variants have been increasingly studied since the last review and some of these have been replicated

in other studies. These represent promising targets for future genetic studies on links with degrees of cachexia. Variants in genes coding for APPR are shown in table 4 in electronic supplementary material.

Central homeostasis

Energy production: The human body responds to stress with dramatic resilience and ultimately aims to maintain homeostasis. The mechanisms to respond to injury can ultimately prove detrimental to the host. For example, although the hepatic acute phase protein response is useful in acute injury (e.g. haemostasis and wound healing), if the response is prolonged and potentially futile (as in advanced cancer), then what results is an accelerated loss of skeletal muscle and excess morbidity and mortality. In certain forms of cancer, patients with cachexia have been observed to have much higher resting energy expenditure (REE) (Fredrix *et al.* 1991). Gene polymorphisms in the regulatory pathways controlling energy intake and expenditure are discussed here. The following section also explores genes involved in growth and development, and metabolic pathways common to both muscle and adipose tissues.

Electronic supplementary table 5 shows the genes involved in energy production, these consist mainly the uncoupling proteins. These are transporters present in the mitochondrial inner membrane that mediate a regulated discharge of the protein gradient that is generated by the respiratory chain. This serves to regulate functions such as thermogenesis, maintenance of the redox balance, or reduction in the production of reactive oxygen species (Ledesma *et al.* 2002). No new functional SNPs were found in this group during this review.

Insulin-like growth factors and related proteins: The IGF signalling pathway consists of two main ligands (IGF-1 and IGF-2), two cell surface receptors (IGFR1 and IGFR2), and six high affinity IGF-binding proteins (IGFBP1–6) (Jones and Clemmons 1995). The predominant regulator of skeletal muscle hypertrophy is through stimulation of the PI3K/AKT pathway by insulin or IGF-1 (Bodine *et al.* 2001a, b; Rommel *et al.* 2001; Glass 2010). Mice in which AKT is transgenically expressed and inducibly activated in skeletal muscle demonstrate dramatic hypertrophy upon the activation signal (Pallafacchina *et al.* 2002; Lai *et al.* 2004; Izumiya *et al.* 2008), helping to prove that AKT is the pathway that is sufficient to mediate hypertrophy downstream of IGF1 upregulation. Activation of AKT leads to an increase in the mTOR/p70S6K pathways and a rise in protein synthesis. As well as inducing protein synthesis, IGF1 can inhibit skeletal muscle atrophy. In the presence of upregulated IGF1 signalling, the atrophy genes *MuRF*-1 and *MAFbx*/*atrogin*-1 are actively inhibited (Bodine *et al.* 2001a, b). A number of new genetic variants for IGF-1 and IGF-1R have been discovered recently and are included (table 6 in electronic supplementary material).

Corticosteroid signalling proteins: Corticosteroids are essential steroid hormones that are secreted by the adrenal cortex and affect multiple organ systems. Corticosteroids are involved in a wide range of physiologic systems such as stress response, immune response and regulation of inflammation, carbohydrate metabolism and protein catabolism. A number of new genes have been included, the corticotropin-releasing hormone receptor 1 (CRHR1) binds to corticotropin-releasing hormone, a potent mediator of endocrine, autonomic, behavioural and immune responses to stress. A number of polymorphisms in this gene have been shown to effect circulating levels of CRP and ICAM-1 (Wilker *et al.* 2009). The glucokinase regulatory protein (GCKR) binds and moves glucokinase (GK); thereby, controlling both activity and intracellular location (Van Schaftingen 1994) of this key enzyme of glucose metabolism (Iynedjian 2009). Genetic variants of this protein have also been linked with alterations in circulating CRP levels (Ridker *et al.* 2008). The genetic variants of the components in the mechanism of corticosteroid signalling are examined in table 7 in electronic supplementary material.

Muscle

In healthy adults, skeletal muscle mass is maintained within relatively narrow limits, reflecting a dynamic balance between protein synthesis and degradation. A predominance of either will result in muscle hypertrophy or atrophy. Even small changes in protein synthesis or degradation will lead to large protein deficits over time due to the continuous process of protein turnover. In cancer cachexia, there is ongoing debate as to whether a reduction in protein synthesis, an increase in protein degradation or a combination of both is more relevant. Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients' function and quality of life and is clearly associated with a poor outcome (Tan *et al.* 2009; Fearon *et al.* 2011; Miller *et al.* 2012). Highlighted here are genetic variations that affect the structure and function of muscle as well as those that regulate muscle synthesis and degradation.

Muscle structure and function: *IL-15* signals through IL-15 receptor alpha (IL-15RA) and is found in abundance in skeletal muscle. *IL-15* is shown to be anabolic, marked by an increase in myosin heavy chain accumulation (Quinn *et al.* 2002). ACTN3 (alpha-actinin 3) binds to actin at the Z-line within muscle fibres and acts to anchor actin filaments. Polymorphisms in ACTN3, IL15 and IL15RA are shown in table 8 in electronic supplementary material.

Recent studies have focussed on polymorphisms associated with alterations of fat free mass in the gene encoding the vitamin D receptor (VDR). Promising new variants encoding for this protein are also included in table 8 in electronic supplementary material. Inhibin β C (*INHBC*) is

a newly added gene, it is part of the transforming growth factor β pathway regulating myostatin (a negative regulator of muscle mass). Polymorphisms identified in this gene are shown in table 8 in electronic supplementary material. Another significant new addition to the genes implicated in cancer cachexia is thyrotropin-releasing hormone receptor (TRHR). Thyrotropin-releasing hormone is released by the anterior pituitary and acts on a number of tissues, including muscle to influence metabolic rates. Genetic variants are listed in table 8 in electronic supplementary material.

New variants in the *ACE* gene have also been included. Alterations in this gene have led to differences in oxygen carrying capacity of muscles as well as increased vasoconstriction in blood vessels within the muscle architecture (Costa *et al.* 2009). Acute and chronic exposure to angiotensin II in animal models are associated with weight loss and enhanced protein breakdown in skeletal muscle (Brink *et al.* 2001). Genetic variants are listed in table 8 in electronic supplementary material.

Muscle synthesis: The main signalling pathway for muscle synthesis is via the IGF-1/PI3K/AKT axis. Polymorphisms relating to these can be found in the insulin-like growth factors and related proteins section (table 6 in electronic supplementary material). mTOR, RUNX1, phosphoinositide 3-kinase (PIK3) and various isoforms have been implicated in hypertrophy signals of muscle mass (Bonetto *et al*. 2011) and have been added to the list of genes. However very few studies have looked into how variants effect structure and function of these gene products. A number of genes from expression arrays were also included as *cis* acting or *trans* acting polymorphisms on the genome may be affecting their expression. Genetic variants influencing function can be found in table 9 in electronic supplementary material.

Muscle proteolysis: In atrophying muscles, the ubiquitin ligases, MuRF1/atrogin-1 and MAFbx, are induced and this response is necessary for rapid atrophy. FOXO isoforms 1 and 3 are known to act on MuRF1 promoter to cause MuRF1 transcription and this leads to dramatic atrophy of myotubes and muscle fibres (Sandri *et al*. 2004; Bonetto *et al*. 2011). IKKα also influences the ubiquitin proteasome pathway (UPP) pathway and a number of functional polymorphisms are listed in table 10 in electronic supplementary material. Another gene encoding for tumour necrosis factor receptor (TNFR) associated factor 6 (TRAF6) is an important adaptor protein involved in receptor mediated activation of various signalling pathways in response to cytokines and bacterial products. TRAF6 also possesses E3 ubiquitin ligase activity causing lysine-63 linked polyubiquitination of target proteins (Paul and Kumar 2011).

A more recent factor, which has captured the attention of many investigators, is the TGF-β family member, myostatin. Genetic null animals for myostatin, demonstrate dramatic muscle hypertrophy (Mosher *et al.* 2007). Myostatin is synthesised and secreted mainly from skeletal muscle cells. Myostatin acts firstly by signalling through the activin type II receptor (ACTRIIB), which then recruits an Alk family kinase, resulting in the activation of a SMAD2 and SMAD3 transcription factor complex (Sartori *et al.* 2009; Trendelenburg *et al.* 2009).

STAT3 has recently been shown to influence muscle wasting by altering the profile of genes expressed and translated in muscle such that amino acids liberated by increased proteolysis in cachexia are synthesized into acute phase proteins and exported into the blood (Bonetto *et al.* 2011).

Peroxisome proliferator activated receptor gamma (PPARγ) and its response gene, Acyl CoA synthetase 5 (*ACSL5*), have an important role in fatty acid metabolism and may affect weight loss in response to caloric restriction. Genetic polymorphisms have demonstrated reduced ACSL5 mRNA in skeletal muscle biopsies (Adamo *et al.* 2007). Polymorphisms in all these genes are presented in table 10 in electronic supplementary material.

Adipose tissue

Incidence of obesity and diabetes continues to increase worldwide. The obese patient is generating a new challenge in medicine and treatment. Physiology of obese patients differs remarkably from a normal weighted individual. Recently, patients suffering from advanced cancer have been found to be overweight rather than underweight (Irigaray *et al.* 2007). This has been shown to confound conventional measurements for risk stratification such as body mass index (BMI). A recent study of pancreatic cancer patients has shown that severe muscle depletion when combined with obesity to be an independent adverse prognostic indicator in this patient group and should be considered as an alternative and more powerful means of risk stratification (Tan *et al.* 2009). It is however unclear how muscle depletion combined with overweight/obesity causes accelerated demise. The adipokines secreted by excess adipose tissue may act as systemic inflammatory mediators, inducing insulin resistance in skeletal muscle and leading to a further increase in muscle protein loss. Increased lipolysis appears to be a key factor underlying fat loss, though decreases in lipid deposition and adipocyte development may also contribute (Legaspi *et al.* 1987). The following section examines polymorphisms in genes regulating adipose tissue metabolism.

Adipogenesis: Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. PPARα is known to participate in the regulation of key proteins involved in extracellular lipid metabolism, fatty acid oxidation and inflammation (Torra *et al.* 2001). PPARγ is an important regulator of fat cell function and involved in differentiation of new adipocytes and by inducing expression of genes promoting uptake of fatty acids,

triglyceride synthesis and insulin sensitivity (Lehrke and Lazar 2005). Lipin proteins (lipin-1, lipin-2 and lipin-3) also act as transcriptional coactivators that regulate expression of lipid metabolism genes (Reue 2009). Polymorphisms in these genes are shown in table 11 in electronic supplementary material.

Lipid turnover and transport: Lipid metabolism protein apolipoprotein C-III (apoC-III) inhibits triglyceride hydrolysis. Genetic variants have been shown to have lower fasting and postprandial serum triglycerides, higher levels of HDL-cholesterol and lower levels of LDL-cholesterol (Pollin *et al.* 2008). A genetic link between lipid metabolism and inflammation has been suggested by the association between variation in the *APOE* gene and plasma CRP. A variant in the *LRRFIP1* gene, which has been implicated in TNFa expression has been shown to be associated with adiposity and inflammation (Plourde *et al.* 2012).

A recent experiment in tumour bearing mice demonstrated during the early and intermediate phases of tumour growth and cachexia, food intake remained normal while plasma levels of proinflammatory cytokines and zinc-α2-glycoprotein rose. The investigators found that genetic ablation of adipose triglyceride lipase (ATGL) prevented an increase in lipolysis and the net mobilization of adipose tissue associated with tumour growth. Unexpectedly, they also observed that skeletal muscle mass was preserved and that activation of proteasomal degradation and apoptotic pathways in muscle was averted. Ablation of hormone sensitive lipase (HSL) had similar but weaker effects. Genetic variants in both these new genes were explored and are listed in table 12 in electronic supplementary material.

Zinc-α-2-glycoprotein (ZAG), otherwise known as LMF, is involved in the specific mobilization of adipose tissue, with increased oxidation of released fatty acids, possibly via induction of uncoupling protein (UCP) expression (Bing *et al.* 2002). ZAG isolated from the MAC16 murine tumour, or from the urine of patents with cancer cachexia, stimulates lipolysis directly through interaction with adenylate cyclase in a guanosine triphosphate (GTP)-dependent process (Bing *et al.* 2004; Bao *et al.* 2005). A polymorphism associated with change of function is shown in table 12 in electronic supplementary material.

Adipokines and adipokine receptors: Adipose tissue, similar to skeletal muscle is an active metabolic and endocrine organ. A number of inflammatory cytokines secreted by adipose tissue have been shown with varying effect to influence the development of diseases such as insulin resistance, diabetes and cancer cachexia by acting on muscle and fat metabolism (Fantuzzi and Faggioni 2000). These adipokines act locally in an autocrine/paracrine manner and/or as endocrine signals to regulate appetite, energy expenditure and a range of physiological processes including insulin sensitivity and inflammatory response which may play an important role in the pathogenesis of cancer cachexia (Kerem *et al.* 2008). Resistin is an adipokine which appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al.* 2006). Polymorphisms within the *RETN* gene which codes for resistin that may influence the development of cachexia (table 13 in electronic supplementary material).

Adiponectin is secreted from adipose tissue and binds to a number of receptors including adiponectin receptors 1 and 2. Adiponectin is produced by the adipocyte and has been shown to decrease insulin resistance. Unlike other adipokines associated with chronic inflammation, adiponectin is inversely related to insulin resistance and BMI. It appears to have protective metabolic and antiinflammatory properties (Marcell *et al.* 2005).

The adipokine, leptin plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. Leptin acts through the leptin receptor. Polymorphisms in genes coding for adiponectin, leptin and their respective receptors are given in table 13 in electronic supplementary material.

Appetite

Muscle mass is clearly sensitive to food intake. The pathogenesis of cancer anorexia is multifactorial and reflects the complexity of the mechanisms controlling energy homeostasis under physiological conditions. The main molecular mechanisms regulating the cancer anorexia–cachexia syndrome include alterations in brain neurochemistry. In particular, the hypothalamic melanocortin system appears not to respond appropriately to peripheral inputs, and its activity is diverted largely towards the promotion of catabolic stimuli promoting metabolism of carbohydrates, lipids, and proteins in peripheral tissues leading to insulin resistance, increased lipolysis and accelerated muscle proteolysis (Tisdale 2002). Ghrelin is produced by the P/D1 cells of the stomach and acts as the natural counterpart to leptin. Ghrelin exerts its effects by promoting food intake (via the orexigenic neuropeptide Y(NPY) system) (Toshinai *et al.* 2003) and decreases sympathetic nerve activity (Matsumura *et al.* 2002). The melanocortin receptors, a family of G-protein coupled receptors, bind α-melanocyte stimulating hormone (α-MSH). Melanocortin 3 and 4 receptors have been found to be involved in feeding behaviour and regulation of metabolism (Fan *et al.* 1997).

NPY acts as a neurotransmitter in the brain and in the autonomic nervous system. An increase in NPY signalling leads to increased food intake (Hanson and Dallman 1995). Genetic variants have been added to this new gene and are listed in table 14 in electronic supplementary material.

Others

Metallothionein (MT) is a family of cysteine-rich, low molecular weight proteins. MTs have the capacity to bind both physiological (such as zinc, copper and selenium) and

xenobiotic (such as cadmium, mercury, silver and arsenic) heavy metals through the thiol group of its cysteine residues, which represents nearly 30% of its amino acidic residues. Zinc homeostasis is often disrupted in cancer cachexia. It has been proposed that the acute phase response can mediate zinc redistribution and accumulation in skeletal muscle tissue and contribute to the activity of the UPP that regulates protein catabolism (Siren and Siren 2010).

P2Y-receptors belong to the superfamily of G-protein coupled receptors and mediate the actions of extracellular nucleotides in cell to cell signalling. The P2Y11 receptor is highly expressed in immunocytes and may play a role in the differentiation of these cells (von Kugelgen 2006). Genetic variants encoding the proteins discussed above may play a role in the development of cachexia and are listed in table 15 in electronic supplementary material.

A number of new genes and SNPs are listed in table 15 in electronic supplementary material, these have evolved from a recent candidate gene approach study identifying SNPs in cancer cachexia. Target SNPs identified from the previous review which could not be genotyped in a cancer population due to experimental design were substituted for the nearest SNP with a linkage disequilibrium of >0.9. These SNPs have been added to the current review as they have been genotyped in patients with cancer cachexia.

Analysis of results

Clinical significance

Clinical significance is defined as any SNP affecting more than one of the recognized hallmarks of cancer cachexia. Inflammation has been shown to influence the severity of cancer cachexia and was therefore identified as a clinical feature. Changes in body composition of muscle and/or fat mass form the basis of cancer cachexia; therefore, SNPs that have the potential to alter an individual's body composition, whether to increase or decrease these components will play a significant clinical role in the development of cancer cachexia or not. Lastly, any SNPs influencing overall survival were included in a definition of clinical significance.

Out of 281 candidate polymorphisms that were identified and summarized here, the functional or clinical significance of 80 polymorphisms have been verified in more than one study. Of these 80 polymorphisms, 24 have been shown to have more than one effect on clinical features associated with cancer cachexia (i.e. inflammation, changes in lean and/or fat mass, and overall survival), these are termed promising SNPs. An indepth analysis of the 24 promising SNPs as biomarkers for susceptibility of cancer cachexia (table 2) is presented below.

(i, ii) The G allele of TLR-1 (−7202A/G) (rs5743551) is associated with elevated TLR1-mediated cytokine production (Wurfel *et al.* 2008; Pino-Yanes *et al.* 2010). TLR1 (−7202G) marks a coding SNP that causes higher TLR1-induced NF-kB activation and higher cell surface TLR1 expression (Wurfel *et al.* 2008). Toll-like receptor (TLR) pathways are critical components of the immune response to pathogens and disease (Trinchieri and Sher 2007). This particular polymorphism has been shown to lead to decreased survival in patients with sepsis (Wurfel *et al.* 2008) and NSCLC (Dai *et al.* 2012). In addition to this SNP in TLR-1, patients carrying the mutant allele T of TLR4 1196C/T (Thr399Ile, rs4986791) had lower TNF-α and sTNFR2 levels compared to patients carrying wild-type alleles (Jermendy *et al.* 2010). These patients carrying the mutant phenotype have also been shown to have increased total body fat, visceral fat, liver fat and decreased insulin sensitivity (Weyrich *et al.* 2010).

(iii) Steps in the inflammatory process include accumulation of lipids, recruitment of leucocytes and smooth muscle cells into vessel walls, and accumulation of extracellular matrix. Intercellular adhesion molecule-1 (ICAM-1) is integral in these cellular processes as interactions between ICAM-1 and activated receptors on the leucocytes result in firm adhesion and transmigration of leucocytes into the basement membrane of the vasculature. The T allele of rs5491 encodes a lysine to methionine substitution in exon 2 in the N-terminal domain of ICAM-1 and results in a protein that is unable to bind to fibrinogen and has a decreased affinity for T cells at lower ICAM-1 concentrations compared to wild-type ICAM-1 (Craig *et al.* 2000). This lead to increased circulating levels of sICAM-1 and an association with insulin resistance and the metabolic syndrome (Hsu *et al.* 2010).

(iv) The C allele of the A37674C *SELP* polymorphism (rs6136) is associated with decreased serum P selectin levels (Miller *et al*. 2004; Volcik *et al*. 2006). P selectin is required for efficient recruitment of neutrophils in acute inflammation and of macrophages in later stages of the inflammatory response and serum levels of P selectin have been found to be significant prognostic factors in survival in patients with gastric and colorectal malignancies (Alexiou *et al.* 2001, 2003). Patients with cancer who carry the C allele of the rs6136 polymorphism in *SELP* gene are at reduced risk of developing cachexia as defined by weight loss >10% (Tan *et al.* 2012).

(v–vii) TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. The –308A allele (rs1800629) has been associated with an increased TNF-α production as well as a six-fold increase in transcription of the *TNF* gene (Wilson *et al.* 1997; Sallakci *et al.* 2005). Women carrying the A/A genotype have been linked to increased fat accumulation (Hoffstedt *et al.* 2000). The −863A allele (rs1800630) associated with decreased transcriptional activity and reduced serum TNF-α levels (Day *et al.* 1998; Skoog *et al.* 1999; Kaluza *et al.* 2000; Sharma *et al.* 2006).

Table 2. Polymorphisms replicated in more than one study and with at least two effects on clinical features associated with cancer cachexia ($n = 24$).

Gene	SNP	Previous S/R	New S/R	Functional Significance	Ancestal allele	SNP allele/s	MAF	SNP type	Systemic inflammation	BMI/ fat mass	Lean mass/ strength	Survival	Repeat studies
TLR-1	rs5743551		✓	The G allele of TLR-1 (-7202A/G) (rs5743551) is associated with elevated TLR1-mediated cytokine production (Wurfel *et al.* 2008) Alleles 7202G and 248Ser, and the 248Ser-602Ile haplotype were associated with circulatory dysfunction among severe septic patients ($0.001 \leq P \leq 0.022$), and with reduced IL-10 ($0.012 \leq P \leq 0.047$) and elevated CRP ($0.011 \leq P \leq 0.036$) serum levels during the first week of sepsis development (Pino-Yanes *et al.* 2010).	C	T	T = 47%	nearGene-5	↑Pino-Yanes *et al.* (2010)			↓Dai *et al.* (2012)	✓Pino-Yanes *et al.* (2010), Wurfel *et al.* (2008), Dai *et al.* (2012)
TLR-4	rs4986791		✓	Serum levels of TNF-alpha and its soluble receptors are elevated and associated with increasing BMI values in obese children. Serum cytokine levels, as modifying factors of insulin, resistance may be affected by TLR4 polymorphisms in obese children (Jermendy *et al.* 2010).	C	T	T = 3%	Missense Thr–Ile	↑Dhiman *et al.* (2008)	↑Weyrich *et al.* (2010)			✓van Rijn *et al.* (2008)
ICAM-1	rs5491		✓	ICAM1 DNA segment variants were associated with sICAM-1 protein level including the novel finding that levels differ by the functional variant rs5491 (Bielinski *et al.* 2011)	A	T	T = 7%	Missense Lys–Met	↑	↑Hsu *et al.* (2010)			✓Hsu *et al.* (2010)
SELP	rs6136	✓		Decreased serum P-selectin levels (Miller *et al.* 2004; Volcik *et al.* 2006) P-selectin genotype is associated with the development of cancer cachexia (Tan *et al.* 2012).	T	G	G = 4%	715 Missense Thr–Pro	↓Miller *et al.* (2004), Volcik *et al.* (2006)			↑	✓Tan *et al.* (2012), Miller *et al.* (2004), Volcik *et al.* (2006)
TNF-α	rs1800629	✓		Increased TNF-α production (Sallakci *et al.* 2005) Six-fold increase in transcription of TNF-α (Wilson *et al.* 1997)	G	A	A = 10%	nearGene-5	↑	↑			✓ Wilson *et al.* (1997), Sallakci *et al.* (2005)
	rs361525		✓	Decreased transcriptional activity (Kaluza *et al.* 2000) Decreased PMBC production of TNF-α after stimulation with T-cell mitogens (Kaluza *et al.* 2000) Decreased insulin resistance (Day *et al.* 1998)	G	A	A = 5%	nearGene-5	↓	↓			✓

Table 2 (*contd*)

Gene	SNP	Previous S/R	New S/R	Functional Significance	Ancestal allele	SNP allele/s	MAF	SNP type	Systemic inflammation	BMI/ fat mass	Lean mass/ strength	Survival	Repeat studies
	rs1800630	✓		Reduced total serum IgE levels (Sharma *et al.* 2006) Reduced serum TNF-α levels (Sharma *et al.* 2006) 31% decrease in transcription of *TNF-α* (Skoog *et al.* 1999)	C	A	A = 15%	nearGene-5	↓	↓			✓
LTA	rs909253	✓		Increased serum TNF-α levels (Stuber *et al.* 1996; McArthur *et al.* 2002)	A	G	G = 40%	Intron	↑			↓	✓
*IL-1*β	rs1143627	✓		Increased expression of *IL-1*β gene with T allele (Lind *et al.* 2007) Increased IL-1β production from whole blood eukocytes after stimulation with LPS with -31T/-l511C/-1470G haplotype (Wen *et al.* 2006) Increased transcriptional activity with −31T/-511C/-1470G haplotype (Wen *et al.* 2006)	G	A	G = 48%	nearGene-5	↑			↑	✓
	rs16944	✓		Increased IL-1β production from whole blood leukocytes after stimulation with LPS with -31T/-511C/-1470G haplotype (Wen *et al.* 2006) Increased transcriptional activity with -31T/-511C/-1470G haplotype (Wen *et al.* 2006) No significant increase in IL-1β production in response to LPS in patients homozygous for T allele Awomoyi *et al.* (2005)	A	G	A = 48%	nearGene-5	↓			↓	✓
	rs1143634	✓		T/T genotype associated with lower plasma levels of IL1-RA (Tolusso *et al.* 2006) Increased human amniochorion IL-1β production after stimulation with LPS (Hernandez-Guerrero *et al.* 2003)	G	A	A = 15%	Synonymous Phe - Phe	↑	↓		↓	✓
IL-6	rs1800795	✓		Lower levels of IL-6 in plasma in healthy subjects (Fishman *et al.* 1998) Higher fasting plasma insulin levels with *G* allele (Yang *et al.* 2005) Lower circulating adiponectin levels with *G* allele (Yang *et al.* 2005)	G	C	C = 19%	nearGene-5	↓	↑	↓		✓
IL-18	rs549908		✓	Increased IL-18 production from LPS and A23187 + PMA stimulated monocytes in 105AA and -137GG (Arimitsu *et al.* 2006) Haplotype of common alleles (GTATA) associated with significantly lower IL-18 (Thompson *et al.* 2007)	T	G	G = 23%	Synonymous Ser - Ser	↑	↓			✓ Arimitsu *et al.* (2006), Thompson *et al.* (2007)

Table 2 (*contd*)

Gene	SNP	Previous S/R	New S/R	Functional Significance	Ancestal allele	SNP allele/s	MAF	SNP type	Systemic inflammation	BMI/ fat mass	Lean mass/ strength	Survival	Repeat studies
IGF-1	rs7136446		✓	Genotype CC of rs7136446 associated with higher body fat and increased maximal force production (Huuskonen *et al.* 2011) Significantly associated with elevated levels of IGF-I (Verheus *et al.* 2008)	T	C	C = 29%	Intron	↑	↑	↑		✓ Verheus *et al.* (2008), Huuskonen *et al.* (2011),
NR3C1	rs6195		✓	Reduced first phase glucose stimulated insulin secretion and disposition index in women, but not in men (van Raalte *et al.* 2012). Associated with enhanced glucocorticoid sensitivity (Russcher *et al.* 2005).	A	G	G = 5%	Missense Asn–Ser	↓	↓			✓ van Raalte *et al.* (2012), Jewell and Cidlowski 2007
GCKR	rs780094		✓	Highly associated with serum CRP levels (Ridker *et al.* 2008)	C	T	T = 39%	Intron	↑	↑			✓ Ridker *et al.* (2008), Stancakova *et al.* (2012)
CNTF	rs1800169		✓	G/A genotype possesses significantly greater muscular strength and muscle quality at relatively fast contraction speeds than do G/G individuals (Roth *et al.* 2001)	G	A	A = 12%	Intron		↑	↑		✓ Roth *et al.* (2001), Heidema *et al.* (2010)
ACSL5	rs2419621		✓	Associated with marked weight loss in dieting and increased levels of ACSL mRNA in skeletal muscle biopsies (Adamo *et al.* 2007; Teng *et al.* 2009)	C	T	T = 26%	nearGene-5		↓	↓		✓ Adamo *et al.* (2007, Teng *et al.* (2009)
LPL	rs328	✓		Significantly lower IL-8 levels (Ak *et al.* 2007) Increased LPL activity Kozaki *et al.* (1993; Groenemeijer *et al.* 1997)	C	G	G = 10%	Stop-Gain 447 Ser - XXX	↓	↓			✓
ZAG	rs4215		✓	GG genotype subjects in rs4215 site have an increased susceptibility to obesity when compared with the AA + AG genotype subjects (Zhu *et al.* 2012) rs4215 was associated with total cholesterol and LDL-C but not with HDL-C or TG (Olofsson *et al.* 2010)	G	A	A = 42%		↑	↑			✓ Zhu *et al.* (2012), Olofsson *et al.* (2010)
RETN	rs1862513	✓		Increased plasma resistin (Cho *et al.* (2004; Osawa *et al.* 2007)	G	C	G = 31%	U/K	↑	↑			✓
ADIPOQ	rs2241766	✓		Increased plasma adiponectin (Berthier *et al.* 2005; Mackevics 2006)	T	G	G = 14%	Cds – synon Gly–Gly		↓	↑		✓
ADIPOR2	rs767870		✓	Increased ADIPOR2 protein in monocytes (Halvatsiotis *et al.* 2010)	A	G	G = 25%	Intron	↑	↑			✓ Vaxillaire *et al.* (2006), Kotronen *et al.* (2009)

Table 2 *(contd)*

Gene	SNP	Previous S/R	New S/R	Functional Significance	Ancestal allele	SNP allele/s	MAF	SNP type	Systemic inflammation	BMI/ fat mass	Lean mass/ strength	Survival	Repeat studies
NPY	rs16139		✓	leucine7 to proline7 in the signal peptide of preproNPY has been associated with increased risk factor for many cardiovascular diseases such as accelerated atherosclerosis (Niskanen *et al.* 2000). Furthermore, elevated serum lipid levels have been associated with the *P7* allele in certain populations (Karvonen *et al.* 2001). Additionally, the L7P polymorphism has been shown to increase the risk of type II diabetes (T2D) as well as earlier onset of the disease and vascular complications (Jaakkola *et al.* 2006).	T	C	C = 6%	Missense Leu–Pro	↑	↑			✓ Karvonen *et al.* (2001), Jaakkola *et al.* (2006).

Obese people express 2.5-fold more TNF mRNA in fat tissue (Hotamisligil 1999). The −238A allele of the SNP rs361525 has shown decreased transcriptional activity of TNFα (Kaluza *et al.* 2000), as well as decreased peripheral mononuclear blood cells (PMBC) production of TNF-α after stimulation with T-cell mitogens (Kaluza *et al.* 2000). It has also been shown to decrease insulin resistance (Day *et al.* 1998).

(viii) LTA is part of the tumour necrosis factor family, it is produced by lymphocytes, and mediates inflammatory responses, stimulation of immune system, and apoptosis (Aggarwal *et al.* 1985). The G allele of the 252 A>G polymorphism (rs909253) has been associated with increased serum TNF-α levels (Stuber *et al.* 1996; McArthur *et al.* 2002), and patients who are A/A homozygotes have been linked with better prognosis in lung cancer and gastric cancer (Shimura *et al.* 1994, 1995).

(ix–xi) IL-1β is a product of the *IL1B* gene and plays a role in potentiating an inflammatory response. The −31 C>T (rs1143627) and −511 C>T (rs16944) polymorphisms in the promoter region of the *IL1B* gene have been linked with increased transcriptional activity of the *IL1B* gene and subsequently increased IL-1β production (Wen *et al.* 2006). These two alleles are also linked with poorer progression free survival and overall survival in advanced gastric cancer (Graziano *et al.* 2005). A synonymous C to T polymorphism at nucleotide position 3953 (rs1143634) has resulted in Increased IL-1β levels (Hernandez-Guerrero *et al.* 2003). The T/T genotype has also been associated with lower plasma levels of IL-1 receptor antagonist (IL-1RA) (Tolusso *et al.* 2006). In addition, the T allele has found to be a major risk factor for cachexia in gastric cancer (Zhang *et al.* 2007), as well as being linked to lower total fat mass (Strandberg *et al.* 2006). The T/T genotype was found to be associated with shorter survival in pancreatic cancer (Barber *et al.* 2000). All of which lead these three polymorphisms to be of particular interest in potential candidates for cancer cachexia.

(xii) IL-6 is a well characterized cytokine involved in a number of cellular functions. IL-6 mediates B cell differentiation and maturation, immunoglobulin secretion, cytotoxic T cell differentiation and acute-phase protein production (Kishimoto 2005). The −174 G>C promoter polymorphism (rs1800795) in the *IL6* gene has been associated with lower serum levels of IL-6 (Fishman *et al.* 1998). The G allele has been linked to higher fasting insulin and lower adiponectin levels which may have a role in the regulation of adiposity (Yang *et al.* 2005). In addition, the C/C genotype has been associated with lower fat free mass and increased waist circumference (Roth *et al.* 2003; Berthier *et al.* 2005).

(xiii) IL-18 is produced by macrophages and functions by binding to the interleukin 18 receptor inducing cell mediated immunity following infection. After stimulation with

IL-18, natural killer (NK) cells and certain T cells release interferon-γ (IFN-γ) that plays an important role in activating the macrophages. Apart from its physiological role, IL-18 is also able to induce severe inflammatory reactions. Individuals with the allele 105AA (rs549908) demonstrated increased IL-18 production from LPS and A23187 + PMA stimulated monocytes (Arimitsu *et al.* 2006). Haplotype of common alleles have been shown to be associated with significantly lower IL-18 (Thompson *et al.* 2007).

(xiv) IGF-1 is one of the most potent natural activators of the AKT signalling pathway which is the main stimulator of cell growth and multiplication. IGF-1 also mediates many of the growth-promoting effects of growth hormone (GH) (Jones and Clemmons 1995). The genotype CC of rs7136446 associated with higher body fat and increased maximal force production (Huuskonen *et al.* 2011), it has also been shown to be significantly associated with elevated levels of circulating IGF-I (Verheus *et al.* 2008).

(xv) The glucocorticoid receptor (GR, or GCR) also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1) is the receptor to which cortisol and other glucocorticoids bind. In the absence of glucocorticoids, GR resides in the cytosol complexed with a variety of proteins including heat shock protein 90 (hsp90), heat shock protein 70 (hsp70) and the protein FKBP52 (FK506-binding protein 52) (Pratt *et al.* 2006). The endogenous glucocorticoid hormone cortisol diffuses through the cell membrane into the cytoplasm and binds to the GR resulting in release of heat shock proteins. Activated GR can bind to the transcription factor NF-κB and prevent it from upregulating target genes (Ray and Prefontaine 1994). The mutant allele of the N363S (rs6195) SNP enhances glucocorticoid sensitivity by increasing gene transcription (Russcher *et al.* 2005). Indeed, in various studies, a link was established between the N363S SNP and characteristics of a cushingoid phenotype, including increased BMI and waist circumference, dyslipidaemia and augmented fasting insulin levels, indicating reduced insulin sensitivity (Roussel *et al.* 2003; Manenschijn *et al.* 2009).

(xvi) The glucokinase regulatory protein (GKRP) also known as glucokinase (hexokinase 4) regulator (GCKR) is a protein produced in hepatocytes. GKRP binds glucokinase (GK), thereby controlling both activity and intracellular location of this key enzyme of glucose metabolism (Van Schaftingen 1994; de la Iglesia *et al.* 1999). The glucose increasing major C allele of rs780094 of GCKR has been shown to be significantly associated with increased insulin resistance leading to development of T2DM and altered lipid metabolism (Stancakova *et al.* 2012; Li *et al.* 2013).

(xvii) CNTF is involved in the neuroendocrine signalling of appetite. It leads to marked weight loss through suppressed food intake without causing hunger or stress (Lambert *et al.* 2001). CNTF receptor-α is abundantly expressed in skeletal muscle (Ip *et al.* 1993; Fraysse *et al.* 2000). As such, recent studies have examined the roles of CNTF and CNTF genotype on neuromuscular disease and muscle function. CNTF administration has been shown to prevent losses of soleus muscle mass and function after hindlimb suspension in rats (Fraysse *et al.* 2000). In humans, the A allele mutation of rs1800169 possesses significantly greater muscular strength and muscle quality at relatively fast contraction speed than the ancestral G allele individual (Roth *et al.* 2001). This polymorphism has also been associated with a global weight gain in healthy humans (Heidema *et al.* 2010).

(xviii) Uptake of FFA by skeletal muscle for metabolism is initiated by transmembrane acyl-CoA synthetase long-chain (ACSL) proteins that esterify FFAs to acyl-coenzyme A (acyl-CoA) molecules. Acyl-CoA species are used mainly in both the synthesis of cellular lipids and the degradation of fatty acids via β-oxidation. Small increases in the expression of ACSL5 in skeletal muscle could have profound effects on FFA utilization (Teng *et al.* 2009). A strong association between the common SNP rs2419621 and rapid weight loss in obese Caucasian females in response to restricted diet has been demonstrated (Adamo *et al.* 2007). The SNP located 12 nucleotides upstream of the second transcription start site of the *ACSL5* gene is characterized by a cytosine (rs2419621) to thymine (rs2419621) transition. This study also demonstrated that the T allele is associated with a 2.2-fold increase of ACSL5 transcript level in skeletal muscle biopsies when compared to noncarriers (Adamo *et al.* 2007). In a further study T allele variants were shown to create a functional *cis*-regulatory E-box element (CANNTG) that is recognized by the myogenic regulatory factor MyoD. The T allele promoted MyoD-dependent activation of a 1089 base pair *ACSL5* promoter fragment in nonmuscle CV1 cells. Differentiation of skeletal myoblasts significantly elevated expression of the *ACSL5* promoter. The T allele variants sustained promoter activity 48 h after differentiation, whereas the C allele variants showed a significant decline. These results revealed a mechanism for elevated transcription of *ACSL5* in skeletal muscle of carriers of the rs2419621 (T) allele, associated with more rapid diet-induced weight loss. This is the first example of a MyoD-binding polymorphism conferring differential promoter activity of a metabolic gene (Teng *et al.* 2009).

(xix) Lipoprotein lipase (LPL) plays a central role in the overall lipid metabolism and transport (Mead *et al.* 2002). The rs328 polymorphism in the *LPL* gene leads to a premature stop codon at amino acid 447. The stop codon results in lower LPL activity (Kozaki *et al.* 1993; Groenemeijer *et al.* 1997), and is associated with lower levels of IL-8 (Ak *et al.* 2007). Individuals not in possession of the stop codon are associated with central obesity (Huang *et al.* 2006).

(xx) Zinc-α-2-glycoprotein (ZAG), otherwise known as LMF, is involved in the specific mobilization of adipose tissue, with increased oxidation of released fatty acids, possibly via induction of uncoupling protein (UCP) expression (Bing *et al.* 2002). LMF isolated from the MAC16 murine tumour, or from the urine of patents with cancer cachexia, stimulated lipolysis directly through interaction with adenylate cyclase in a guanosine triphosphate (GTP) dependent process (Bing *et al.* 2004; Bao *et al.* 2005). This effect was also produced by the interaction of LMF with the β_3-adrenoceptor (Russell *et al.* 2002). Genotypes of rs4215 in *ZAG* gene have been suggested to be significantly associated with obesity. The GG genotype subjects in rs4215 site have an increased susceptibility to obesity when compared with the AA+AG genotype subjects (Zhu *et al.* 2012). In a separate study variations in the rs4215 genotype have been linked with changes in circulating levels of total cholesterol and LDL-C.

(xxi) The adipokine resistin potentiates a proinflammatory state, resistin also appears to have effects on substrate metabolism through impairment of insulin action and insulin independent pathways (McTernan *et al.* 2006). The –420 C>G polymorphism (rs1862513) is shown to be linked to increased plasma resistin (Cho *et al.* 2004; Osawa *et al.* 2007), and individuals with the G/G genotype are associated with an increased prevalence of obesity (Norata *et al*. 2007). Overall, increased plasma resistin has shown to correlate with increased CRP and insulin resistance (Degawa-Yamauchi *et al.* 2003; Silswal *et al.* 2005; Nagaev *et al.* 2006; Kusminski *et al.* 2007; Osawa *et al.* 2007).

(xxii, xxiii) Adiponectin is a protein hormone that is exclusively secreted from adipose tissue and modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism (Diez and Iglesias 2003). The *ADIPOQ* gene, which codes adiponectin, has a 45 T>G polymorphism (rs2241766) that is associated with increased plasma adiponectin (Berthier *et al.* 2005; Mackevics *et al.* 2006). Individuals with G/G genotype have been observed to be leaner with less abdominal fat (Loos *et al.* 2007). The adiponectin receptors ADIPOR2, serves as a receptor for adiponectin and mediates increased AMPK and PPAR-α ligand activities, as well as fatty acid oxidation and glucose uptake by adiponectin (Yamauchi *et al.* 2003). In peripheral monocytes, carriers of the major A allele (homozygotes and heterozygotes) of rs767870 polymorphism had higher levels of ADIPOR2 protein expression compared to homozygotes of the minor G allele (Halvatsiotis *et al.* 2010). This same SNP has also been associated with liver fat content and the incidence of type II DM (Vaxillaire *et al.* 2006; Kotronen *et al.* 2009).

(xxiv) Proinflammatory cytokines (TNFα and IL-1β) and hypothalamic serotonergic neurons have been implicated in the dysfunction of the hypothalamic melanocortin system (Inui 1999). The orexigenic neuropeptide Y (NPY) peptide system appears to be strongly influential in the control of feeding (Plata-Salaman 2000). The pathway originates in the hypothalamic arcuate nucleus (ARC) and extends projections widely over the brain (Plata-Salaman 2000). The role of cytokines in cancer anorexia may be affected through influence on the NPY system. The genetic variant rs16139 causing, leucine7 to proline7 in the signal peptide of preproNPY has been associated with increased risk factor for many cardiovascular diseases such as accelerated atherosclerosis (Niskanen *et al.* 2000). Further, elevated serum lipid levels have been associated with the P7 allele in certain populations (Karvonen *et al.* 2001). Additionally, the L7P polymorphism has been shown to increase the risk of type II diabetes as well as earlier onset of the disease and vascular complications (Jaakkola *et al.* 2006).

Pathway analysis

The 80 polymorphisms which have been verified in more than one study were found across 51 genes. These genes were entered into the IPA algorithm as focus genes and were found to be significantly interconnected in two major networks (table 3). The two networks are presented in figures 1 and 2.

Putative functions

Since gene association studies often identify surrogates for putative causal SNPs, it is imperative that data from selected SNPs be subjected to further analysis using prediction tools to shortlist candidates for finer analysis before causality could be established. Unless causative SNPs/genes are identified, development of targeted therapeutics are difficult to achieve. Of the 42 nonsynonymous polymorphisms entered into the SIFT algorithm (table 16 in electronic supplementary material) seven SNPs had a significant score ($P < 0.05$) causing an intolerant change of amino acid. Since SIFT programme only evaluates nonsynonymous SNPs, and the majority of polymorphisms lie within the regulatory regions at 3′, 5′ ends and in introns, we interrogated the SNPs selected using the SCAN database for insights into the potential contribution of selected SNPs on gene regulatory functions (*cis* effects or *trans* effects, eQTLs) (Hunter and Crawford 2008; Schadt *et al.* 2008; Fehrmann *et al.* 2011; Hao *et al.* 2012; He *et al.* 2013). We identified 132 SNPs as potential eQTLs in HapMap study populations (Caucasian, Yoruban, Han Chinese/Japanese) (table 17 in electronic supplementary material). Analysis of selected candidate SNPs for potential eQTLs revealed several target genes that are regulated both in *cis* and *trans*, as expected. Fine regulation in complex biological networks by a SNP may be direct or indirect, and likely influence gene expression through short or long range interactions. Prediction for regulation of expression was limited to those showing high statistical significance (P value $< 10^{-4}$) and to any one of

Table 3. Ingenuity pathway analysis of genes that were replicated in more than one study ($n = 51$).

Network	Molecules	Calculated score	Focus genes	Top functions of network
1	ADCY, **ADRB1, ADRB2, ADRB3**, beta arrestin, **CCL2**, **CRHR1**, cytochrome c, endothelin, estrogen receptor, G protein, G protein alphai, G protein β, GNRH, Gpcr, Gs-coupled receptor, **IGF1R**, IL-17f dimer, **IL-6R**, Jnk, **LEP, MC3R, MC4R,** Mmp, NADPH oxidase, **NPY**, P110, p85 (pik3r), Pdgfr, PLC, Shc, **TRHR**, trypsin, TSH, Voltage gated calcium channel	22	12	Cell signalling, neurological disease, nutritional disease
2	Three BETA HSD, **ADIPOQ, ADIPOR2,** Akt, AMPK, **AZGP1,** FKHR, **FOXO1, GHRL,** Gm-csf, HLA-DQ, Igf, **IGFBP3,** IL-8r, jINK1/2, MTORC1, Na+, K+ - ATPase, Nr1h, Pde4, PEPCK, **PIK3CB,** Ppp2c, PRKAA, Proinsulin, Ptk, Rab5, Rxr, Scavenger receptor class A, sPla2, T3-TR-RXR, thymidine kinase, **UCP2, UCP3, VDR,** vitaminD3-VDR-RXR	20	10	Endocrine system, development and function, carbohydrate metabolism, molecular transport

Focus genes are in bold. The software illustrates networks graphically and calculates a score for each network, which represents the approximate 'fit' between the eligible focus molecules and each network. The network score is based on the hypergeometric distribution and is reported as the –log (Fisher's exact test result).

the HapMap populations. Promising candidates from SCAN database should be further validated by independent methods (RT-PCR) to confirm for direction and magnitude of expression changes in a tissue-specific manner. In addition to pathway-based-candidate SNP approaches for cachexia, genomewide association studies (GWAS) have the potential to identify promising variants for further interrogation of the genome for genetic predisposition. International efforts are underway from our group to conduct large scale association studies using well defined phenotypes of cachexia and higher sample size to achieve the needed statistical power.

Discussion

Even with the same tumour type and burden, one individual may become cachectic, whereas another will not, such variation may relate to host genotype. Genetic variation in immunity and associated signalling pathways is known to relate to outcomes in major sepsis (Thair *et al.* 2011), and recent findings suggest a similar pattern in cancer cachexia (Tan *et al.* 2012). SNP in the IL-1, IL-6, and IL-10 genes that are linked to production rates of these cytokines have been associated with the prevalence of cachexia in gastric or pancreatic cancer (Tan and Fearon 2010). For example, the 1082G allele in the IL-10 promoter has been validated as a procachectic genotype in an independent cohort (Deans *et al.* 2009; Sun *et al.* 2010a, b). IL-10 has been shown to be elevated in a Myc/mTOR driven murine model of cancer cachexia (Robert *et al.* 2012), as well as in cachectic patients with colorectal cancer (Shibata *et al.* 1996). Others have identified associations with cachexia defined as >10% weight loss and polymorphisms in cytokine genes such as the IL1-B 3954C/T polymorphism (rs1143634) in patients with gastric cancer (Zhang *et al.* 2007). Cancer-related anorexia has been associated with the TNF-308G/A polymorphism (rs1800629) in patients with non-small cell lung cancer (Jatoi *et al.* 2010). Most recently, the C allele of the rs6136 polymorphism in the p-selectin gene has recently been associated with weight loss in a large heterogenous group of cancer patients and validated in an independent cohort (Tan *et al.* 2012). Taken together, these findings are consistent with a key role for the immune system in the variable presentation of cachexia. However, cancer cachexia has been defined in a number of ways (Bozzetti and Mariani 2009; Argiles *et al.* 2010; Muscaritoli *et al.* 2010; Argiles *et al.* 2011; Baracos 2011), mainly focussed on weight loss and the presence of systemic inflammation. This has led to phenotyping in many of the existing studies on genetic association relying wholly on a degree of weight loss and inflammation. However, one recent definition described cancer cachexia with a more musculocentric view (Fearon *et al.* 2011). This definition highlights the importance of skeletal muscle loss as one of the most significant events in cachexia and is associated with a poor

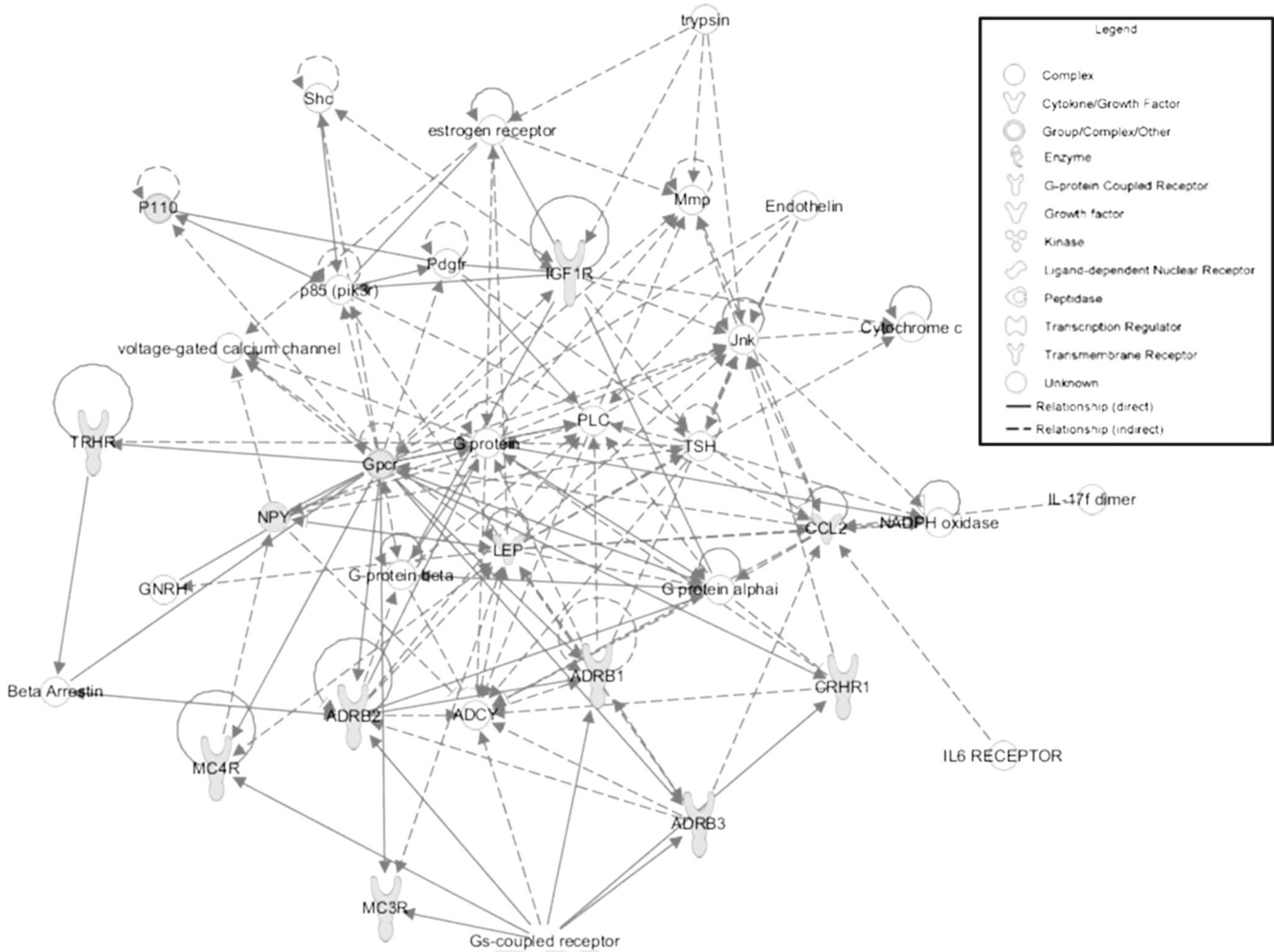

Figure 1. Connection map for first ranked network. Genes with variants that had functional or clinical associations replicated in at least one study were entered into the ingenuity pathway analysis software for an unsupervized functional analysis to discern regulatory networks that involved these molecules. Focus genes are shaded in grey. Solid lines show direct interaction (binding/physical contact); dashed lines show indirect interaction that is supported by the literature but possibly involving ≥1 intermediate molecules that have not been investigated definitively. Molecular interactions that involved only binding are connected with a line without an arrowhead because directionality cannot be inferred.

outcome (Tan *et al.* 2009; Fearon *et al.* 2011). In genetic studies of common diseases, the capacity to define genotypes is often far better than the capacity to define phenotypes; therefore, more robust classifications of phenotypes need to be sought. With the existing studies focussing mainly on weight loss and CRP to define cancer cachexia phenotypes this may have led to the discovery of associated SNPs exclusively involved in mediating the immune response. Recent advances in the use of routine CT scans in a cancer patient's care have led to unmasking of detailed body composition analysis for accurate measurements of skeletal muscle and adipose tissue mass (Prado *et al.* 2009). Taking into account, a level of skeletal muscle mass alongside degrees of weight loss and inflammation will improve accuracy of phenotyping and may open up the analysis to include genetic variants in muscle specific genes.

While some genetic variants can predisopose individuals to develop cancer cachexia, also there will be variants which may protect against the devlopment of the condition. In multigene studies, judicious selection of candidate genes and polymorphisms within them is a key element of study design. It is always important to choose genes products which interact within regulatory or metabolic pathways. In most cases, it is not realistic to analyse all possible gene variants and combinations; hence, existing polymorphisms should be initially prioritized on the basis of their likelihood to affect function of the encoded product (Tabor *et al.* 2002). Published studies linking genetic variation to cancer cachexia are listed in table 1, the definitions of the cancer cachexia phenotype vary greatly from weight loss cut-offs and inclusion of systemic inflammation, to performance status assessment and quality of life scores. The only study to directly state and include an independent validation cohort to confirm the presence of a significant SNP is the detection of the p-selectin genotype from our group (Tan *et al.* 2012). In the discovery cohort, a further 20 significant SNPs were

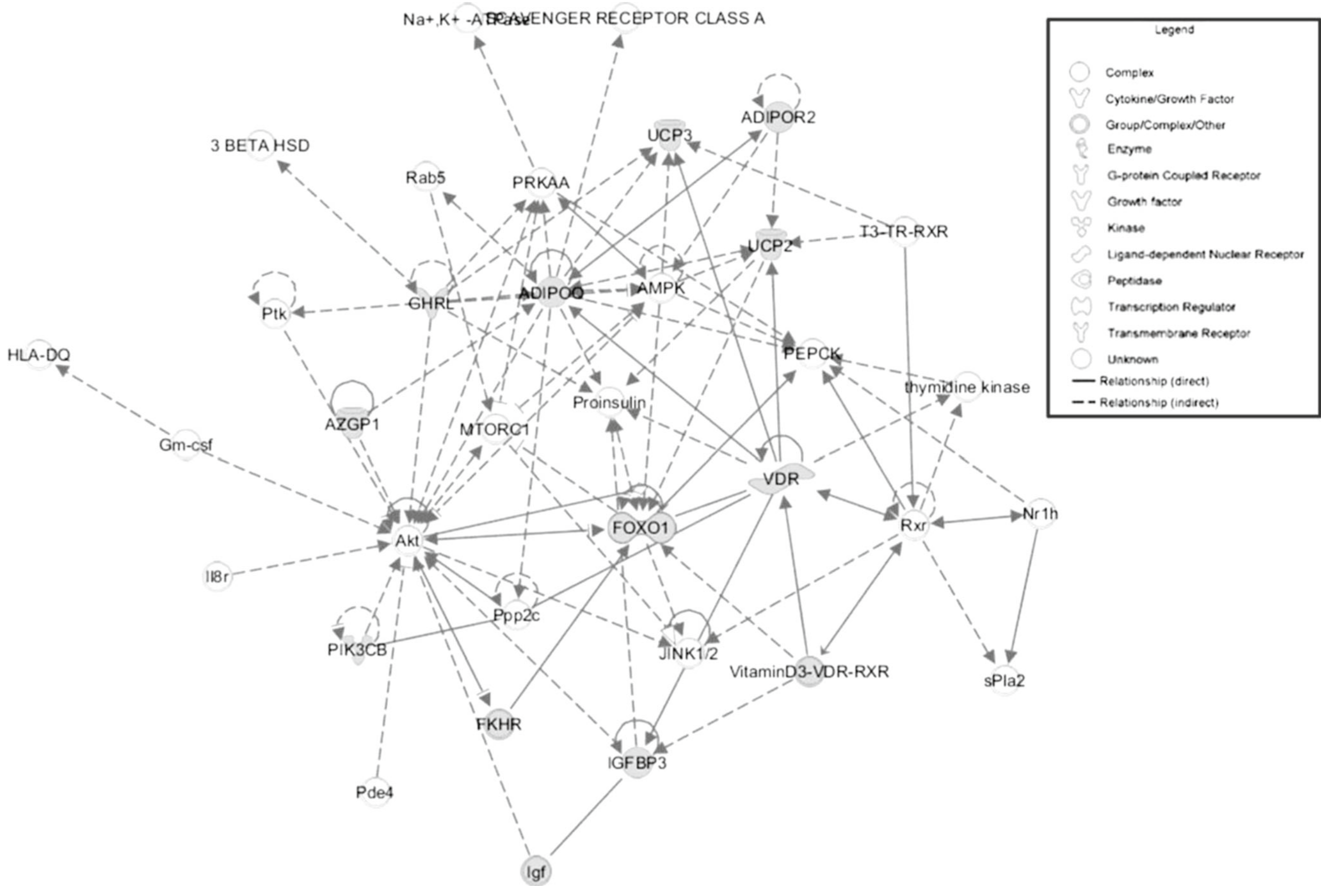

Figure 2. Connection map for second ranked network. Genes with variants that had functional or clinical associations replicated in at least one study were entered into the ingenuity pathway analysis software for an unsupervized functional analysis to discern regulatory networks that involved these molecules. Focus genes are shaded in grey. Solid lines show direct interaction (binding/physical contact); dashed lines show indirect interaction that is supported by the literature but possibly involving ≥ 1 intermediate molecules that have not been investigated definitively. Molecular interactions that involved only binding are connected with a line without an arrowhead because directionality cannot be inferred.

found; however, these did not reach significance in the validation cohort. These SNPs may be significant, but due to the size of the independent validation cohort, they failed to reach significance.

In the current review, functional polymorphisms in genes with a possible role in cachexia have been recorded as well as polymorphisms with clinical consequences related to cachexia such as inflammation, weight/body composition changes and cancer survival. Since cancer cachexia is a multifactorial disease involving a variety of biological pathways, it can be assumed that analysis of combinations of gene variants encoding interacting factors within a biological chain or cascade, rather than isolated investigation of its single components, may have more chances to reveal real causative connections between gene polymorphisms and phenotypes. Of the 80 polymorphisms with a potential role in the development of cachexia that have been independently verified in at least one repeat study, 24 polymorphisms have been shown to have more than one effect on clinical features associated with cancer cachexia. These 24 polymorphisms are likely to be the most promising candidates in terms of susceptibility biomarkers of cancer cachexia and should be further investigated.

Eighty-eight newly identified genes with a role in cancer cachexia were included since the last review. However, the main limitation to identifying new SNPs in these genes was the lack of studies to date into functional polymorphisms within them. Undertaking a GWAS would be one way to overcome this potential limitation and in the future would be preferable to repeating candidate gene selection studies, however without an adequate sample size and highly accurate phenotyping, coupled with lack of government funding to complete the project, candidate gene association studies will continue to provide novel insights into the genetics of cancer cachexia.

In conclusion, the current review has expanded on an initial framework to further enhance the possibility of identifying functional polymorphisms involved in cancer cachexia. Based on the expansion of the definition of cancer cachexia along with an inclusion of skeletal muscle mass and not just

weight loss and systemic inflammation, new muscle specific SNPs may provide novel biomarkers in the early detection of individuals at risk of developing cancer cachexia.

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Clinical Classification of Cancer Cachexia: Phenotypic Correlates in Human Skeletal Muscle

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Abstract

Background: Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, and duration of survival. One impediment towards the effective treatment of cachexia is a validated classification system.

Methods: 41 patients with resectable upper gastrointestinal (GI) or pancreatic cancer underwent characterisation for cachexia based on weight-loss (WL) and/or low muscularity (LM). Four diagnostic criteria were used >5%WL, >10%WL, LM, and LM+>2%WL. All patients underwent biopsy of the rectus muscle. Analysis included immunohistochemistry for fibre size and type, protein and nucleic acid concentration, Western blots for markers of autophagy, SMAD signalling, and inflammation.

Findings: Compared with non-cachectic cancer patients, patients with LM or LM+>2%WL, mean muscle fibre diameter was reduced by about 25% ($p = 0.02$ and $p = 0.001$ respectively). No significant difference in fibre diameter was observed if patients had WL alone. Regardless of classification, there was no difference in fibre number or proportion of fibre type across all myosin heavy chain isoforms. Mean muscle protein content was reduced and the ratio of RNA/DNA decreased in patients with either >5%WL or LM+>2%WL. Compared with non-cachectic patients, SMAD3 protein levels were increased in patients with >5%WL ($p = 0.022$) and with >10%WL, beclin ($p = 0.05$) and ATG5 ($p = 0.01$) protein levels were increased. There were no differences in phospho-NFkB or phospho-STAT3 levels across any of the groups.

Conclusion: Muscle fibre size, biochemical composition and pathway phenotype can vary according to whether the diagnostic criteria for cachexia are based on weight loss alone, a measure of low muscularity alone or a combination of the two. For intervention trials where the primary end-point is a change in muscle mass or function, use of combined diagnostic criteria may allow identification of a more homogeneous patient cohort, reduce the sample size required and enhance the time scale within which trials can be conducted.

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Introduction

Cancer cachexia has been defined recently 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 [1]. Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life and duration of survival. Skeletal muscle loss appears to be the most significant event in cancer cachexia and is associated with a poor outcome [1,2]. The international consensus on the classification of cancer cachexia suggested that diagnostic criteria should take into account not only that weight loss is a signal event of the cachectic process but that the initial reserve of the patient should also be considered (either low BMI or low level of muscularity). Although the latter concept has some validation in terms of clinical risk [2], there has been no evaluation of the biological correlates in terms of changes within skeletal muscle itself.

Human skeletal muscle is composed of muscle fibres that are classified depending on their speed of contraction and predominant type of energy metabolism. Muscle fibres can be classified as type I (slow-twitch) and type II (fast-twitch) fibres based on their

predominant myosin heavy chain (MyHC) isoform content. Generally, type I and type IIa fibres utilise oxidative phosphorylation, whereas type IIx and IIb fibres harness primarily anaerobic metabolism to generate ATP. Both the percentage and structural morphology of the fibre type will determine the phenotypic capacity and functional performance of any given muscle. Environmental factors in both health and disease have a direct impact leading to changes in fibre type/morphology and consequent functionality; such processes include aging, exercise, chronic disease, and cachexia [3–7]. The change, preservation or loss of fibres may influence clinical symptoms and there is some evidence that all types of MyHC is targeted selectively in cancer cachexia [8]. Ongoing loss of protein in muscle tissue may lead to muscle fibre shrinkage and a reduction in cross-sectional area (CSA). Equally, loss of muscle fibre CSA may lead to loss of aerobic capacity (VO_2 max) in healthy subjects as well as cancer patients [5,9].

Although systemic inflammation is generally thought to be an important upstream mediator of cancer cachexia [10], the precise molecular mechanisms that mediate the changes in protein synthesis and degradation that ultimately lead to atrophy of muscle fibres in cancer cachexia in humans are not known. For each animal model that has been studied, different pathways have been implicated. From such animal models there is a predominant impression that increased degradation via activation of the ubiquitin proteasome pathway (UPP) is important [10]. In contrast, human data is very limited. Activation of protein degradation via the UPP has not been a consistent finding [11] [12]. This has led to suggestions that autophagy may be important or that pathways that may influence both synthesis and degradation may be important (e.g. TGF-β/SMAD signalling) [13].

In the present study we chose to evaluate the relationship between the different cachexia definitions, systemic inflammation (serum C-reactive protein) and potential inflammatory signalling pathways within muscle (phospho-STAT3 and phospho-NFkB). We also examined for potential associations between the various cachexia definitions and activation of autophagy pathways or TGF-β/SMAD signalling.

The aim of this study was to investigate the changes in muscle fibre biology with regards to morphological structure and composition, to study alteration in various pathways that may account for altered fibre size and relate these changes to the different diagnostic criteria that have been proposed as part of the recent international consensus on the classification of cancer cachexia [1].

Materials and Methods

Patient Recruitment, Identification, Consent and Ethics

Patients with resectable disease and suitable for the study were identified via the upper gastrointestinal cancer multi-disciplinary team (MDT) meetings at the Royal Infirmary, Edinburgh, UK. Written consent was given prior to entry into the study. All procedures were approved by the NHS Lothian local research ethics committee. The study conformed to the standards set by the Declaration of Helsinki.

Calculation of Weight Loss

Pre-morbid weight was recalled by the patient and verified where possible from the medical notes. Although there may be recall bias, evidence to support the reliability of self-reported weight and weight history [14,15] is well documented. Individual weight loss was calculated and expressed as percentage of pre-morbid body weight lost.

Classification of Cancer Cachexia

I. Weight loss >5% over past 6 months (in absence of simple starvation) (WL>5%)

II. Weight loss >10% over past 6 months (in absence of simple starvation) (WL>10%)

III. Stature adjusted skeletal muscle index consistent with low muscularity (LM) (see 'CT-image analysis' for cut-offs)

IV. Stature adjusted skeletal muscle index consistent with low muscularity and any degree of weight loss >2% (LM + > 2%WL)

Rectus Abdominis Muscle Biopsy and Storage For Biochemical Analysis

All biopsies were taken at the start of open abdominal surgery under general anaesthesia. Patients had fasted overnight prior to surgery. The edge of the rectus abdominis was exposed and a 1 cm^3 specimen removed using sharp dissection. The biopsy was cleaned of gross blood contamination. Obvious fat/fibrous tissue was removed prior to placement in a cryotube and being snap frozen in liquid nitrogen and stored at $-80°C$.

Rectus Abdominis Muscle Sample Preparation for Cryo-Section

A 0.1–0.5 cm^3 section of muscle was cut. Liquid nitrogen was used to cool isopentane solvent in a tube to a temperature of $\sim-190°C$. The section of muscle was stitched onto a segment of cork. OCT solution was placed at the junction between the cork base and the muscle. This was then lowered with the cork uppermost (i.e. muscle first) into cooled solvent and held for approximately 5 minutes (until the muscle was frozen). Samples were then stored at $-80°C$ until use.

CT Image Analysis

CT scans used for the analysis were done solely for routine cancer care. A transverse CT image from the third lumbar vertebrae (L3) was assessed for each scan date and tissue volumes estimated [16]. All CT images were analysed by a single trained observer. Cross-sectional area for muscle and adipose tissue was normalized for stature (cm^2/m^2).

Estimates of whole body stores were generated from the raw data (cm^2) using the regression equations by Mourtzakis et al. [17], which show a close correlation between muscle and fat areas in CT images at the third lumbar vertebrae and whole body compartments of fat-free mass (FFM) and fat mass (FM) respectively.

$$\begin{aligned}&\mathit{Total\ body\ fat\ free\ mass}(FFM)(kg)\\&=0.3\times\left[\mathit{skeletal\ muscle\ at\ L3}\left(cm^2\right)\right]+6.06(r=0.94)\end{aligned}$$

$$\begin{aligned}&\mathit{Total\ body\ fat\ mass}(FM)(kg)\\&=0.042\times\left[\mathit{total\ adipose\ tissue\ at\ L3}\left(cm^2\right)\right]+11.2(r=0.88)\end{aligned}$$

The respective indexes for FFM and FM (kg/m^2) were calculated.

Cutoffs for low muscularity were based on a CT-based sarcopenic obesity study of cancer patients by Prado et al. (i.e.,

L3 skeletal muscle index: $\leq 38.5\ cm^2/m^2$ for women and $\leq 52.4\ cm^2/m^2$ for men) [18].

CT scans used were routine diagnostic staging CT scans which were performed within 30 days of a diagnosis of cancer and all were in treatment naive patients. The median time to biopsy after the CT scan was 18 days.

Immunohistochemistry

The frozen muscle sections were co-stained for laminin (L9393, Sigma-Aldrich, Buchs, Switzerland) and myosin heavy chain type I or IIa to distinguish each fibre type (BA-D5 for type I, SC-71 for type IIa). The paraffin sections were stained for phospho-STAT3 (D3A7, Cell Signaling Technologies, Danvers, MA, USA) with a Ventana discovery XT (Roche group, Tucson, USA). Images of the entire tissue section were acquired using a VS120 slide scanner (Olympus Corporation, Tokyo, Japan). The distribution of myosin heavy chain fibre types, the cross section area of the individual fibres in the section, and the phospho-STAT3 positive nuclei and staining density were analysed using the proprietary image analysis platform ASTORIA (Automated Stored Image Analysis) developed by Novartis/Preclinical Safety.

Tissue Preparation for DNA, RNA and Protein Extractions

Skeletal muscle tissue was minced and ground on dry ice. Aliquots were weighed using an analytical balance (Mettler Toledo) and stored at −80°C until use.

DNA and RNA Extraction and Linearity of the Extraction Method

DNA and RNA from human skeletal muscle tissue was extracted and purified with the automated Maxwell 16 system (Promega, Duebendorf, Switzerland). To determine the linearity of the extraction methods using the Maxwell 16 system, DNA and RNA was extracted from 4 mg, 6 mg, 8 mg, and 10 mg of muscle, respectively. Calculating the total DNA and RNA content per wet weight (which in a linear extraction system should be equal for all aliquots), allowed us to define the linear range of the Maxwell 16 extraction system. Based on these preliminary studies, aliquots of 4–8 mg human skeletal muscle tissue were used for all subsequent DNA and RNA extractions. Using more starting material drastically reduced the total DNA and RNA content per wet weight (data not shown).

For DNA extraction, the Maxwell 16 LEV Blood DNA Kit (Promega) was used with a slightly adapted protocol compared with the manual's instructions. Briefly, 300 µl of Tail Lysis Buffer from the kit ReliaPrep gDNA Tissue Miniprep System (Promega) was added to minced and ground human skeletal muscle tissue in Precellys 24 lysing kit tubes. Tissue was further homogenized using the high-throughput homogenizer Precellys 24, for 10 s. After cooling on ice for 5 minutes, 30 µl of the protein K and 5 µl of the 1-Thiolglycerol solution were added. This mixture was incubated at 56°C for 2 hrs. Afterwards, the lysate was transferred into well 1 of the LEV Blood DNA cartridge, and diluted with 300 µl nuclease-free water. For the elution, 50 µl of elution buffer was added into elution tubes. The Maxwell 16 instrument was started using the DNA Blood program.

For RNA extraction, the Maxwell 16 LEV simplyRNA Tissue Kit was used (Promega), following the manual's instructions. Briefly, minced and ground human muscle tissue was incubated in 200 µl of chilled 1-Thioglycerol/Homogenization solution and further homogenized using the Precellys 24 system (see DNA). Afterwards, the samples were heated at 70°C for 2 min, then the lysates were allowed to cool down. 200 µl of lysis buffer was added to the cooled-down homogenate, mixed vigorously, followed by transfer of the total 400 µl into well 1 of the Maxwell 16 LEV cartridge. 5 µl of DNAse was added to well 4 of the cartridge and, 50 µl RNAse-free water was added to 0.5 ml Elution Tubes and the RNA extraction program was started at the Maxwell 16 instrument.

Extracted DNA and RNA were measured spectrometrically using a Trinean DropSense Instrument (Trinean, Gentbrugge, Belgium) for quantity and quality.

Protein Extractions

To extract proteins, 300 µl of PhosphoSafe Extraction Reagent (Millipore) was added to a specific amount (between 8 and 18 mg) of homogenized human skeletal muscle tissue. To further homogenize the samples, the Precellys 24 system was used (see section above). After incubation on ice for 5 min, the lysates were spun at 800×g for 5 min at 4°C. Supernatants were transferred into new tubes and spun for another 12 min at 1600×g at 4°C. Supernatants were collected and protein concentrations measured using the BCA Protein Assay Kit (Pierce) with BSA as a standard. Afterwards, phosphatase inhibitor cocktail (Roche) was added and the samples were stored at −80°C until further use.

Western Blots

20 µg of human skeletal muscle protein extracts (see above) in reducing Laemmli SDS sample buffer were boiled for 5 min at 95°C and then separated by SDS-PAGE on 4–20% gradient gels (Bio-Rad, Cressier, Switzerland), blotted to Nitrocellulose membranes (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad), blocked for 1 h in 5% non-fat milk in Tris-buffered saline+0.05% Tween-20, incubated overnight with primary antibody, rinsed, and incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Santa Cruz, Heidelberg, Germany) at room temperature. Blots were developed using ECL (Roche, Rotkreuz, Switzerland) or SuperSignal West Femto substrate (Thermo Scientific, Wohlen, Switzerland) and exposed to Kodak film (Kodak, Rochester, NY, USA).

Rabbit monoclonal antibodies used were: Beclin-1 (clone D40C5), Atg5 (clone D1G9) , Atg7 (clone D12B11), Atg12 (clone D88H11), SMAD3 (clone C67H9), phospho-NFκB p65 (Ser536) (clone 93H1) and α-tubulin (clone 11H10) (all from Cell Signaling Technologies, Danvers, MA, USA), phospho-SMAD3 (Ser423/Ser425, clone EP823Y) (Millipore, Billerica, MA, USA). Rabbit polyclonal antibodies used were: Gelsolin (Cell Signaling Technologies).

Western blots were analyzed densitometrically using ImageJ software version 1.45 (NIH, Bethesda, MD, USA; http://rsbweb.nih.gov/ij). Band intensity of each sample was normalised to that of α-tubulin.

C - Reactive Protein (CRP)

Serum CRP concentration was measured with an automated immunoturbidimetric assay by clinical chemistry department, Royal infirmary Edinburgh, using blood collected from patients at the time of recruitment and before any therapeutic intervention.

Statistical Analysis

Results are expressed as mean (± SEM). Comparisons between groups were performed using unpaired Student's t tests, whereas possible relationships were evaluated using Pearson's correlations. Results were considered significant if p values were less than 0.05. The program SPSS (version 20, SPSS, Chicago, IL, USA) was used for all the statistical tests.

Results

Patient Demographics

A total of 41 cancer patients with resectable UGI or pancreatic cancer were recruited. In general, patients were over 65 years of age, predominantly male and had sustained, on average, 5% loss of weight compared with pre-illness levels (Table 1). Patients were grouped based upon the concepts of the International Classification Framework [1] according to weight loss or weight loss in association with low muscularity. The specific phenotypes considered were weight loss >5% (WL>5%), weight loss >10% (WL>10%), low muscularity (LM), and LM with weight loss >2% (LM+>2% WL). Although BMI was reduced in all groups classified as cachectic, only the LM and LM+>2% WL groups had a significantly lower fat free mass index (Table 1).

Muscle Fibre Size, Number, and Type

If patients were classified as cachectic by LM or LM + >2%WL, fibre size was reduced significantly (all types of myosin heavy chain fibre) when compared with non-cachectic patients and controls (Figure 1A). The association of cachexia with reduced fibre size was not observed if patients were classified according to WL alone. Representative immunohistological sections demonstrating differences in fibre diameter between a healthy control and an individual in Group II versus Group IV is shown in Figure 1B. Immunohistology for type I and IIa resulted in complementary staining in general, whereas fibre type IIb resulted in very low staining intensity as reported elsewhere [19]; therefore quantitative analysis was done only with type I and IIa but not with type IIb (Table 2). As would be expected from a decrease in fibre size, there was a trend across all groups for fibre density to increase in those with cachexia. However, due to large variability, this was not statistically significant. There was no evidence of selective fibre atrophy across any of the classification groups (Table 2).

Protein Content

The results for skeletal muscle protein content are shown in Figure 2(A). When compared with non-cachectic patients, muscle protein content was reduced significantly (approximately 13%) in patients with either >5% WL or LM + >2%WL (Figure 2A and table 3). However if the LM criteria were applied alone no difference in the protein content was observed. In addition, patients with >10% WL showed a 10% reduction in protein content when compared with non-cachectic patients but this difference did not reach statistical significance (Figure 2A and table 3).

RNA, DNA, and RNA/DNA Ratio

The results for skeletal muscle DNA and RNA content are also shown in Figure 2(B,C, and D) and table 3. RNA content was not significantly different in cachectic patients when compared with non-cachectic patients according to any of the diagnostic criteria (Figure 2B and table 3). In contrast, DNA content was increased by 50% with >5% WL but decreased by ~40% in patients with LM (Figure 2C). The ratio of RNA/DNA was decreased (approximately 30%) in patients with >5% WL and LM + >2%WL (Figure 2D).

Autophagy Pathways

In patients with >10% WL, Beclin and ATG5 protein levels were increased significantly in cachectic patients when compared with non-cachectic patients (Figure 3). ATG7 and 12 levels were not different in cachectic patients when compared with non

Table 1. Demographic data of the patients involved in the study.

	All Patients (n = 41)	Weight Stable (n = 23)	Weight Loss >5% (n = 18)	p	Weight Stable (n = 30)	Weight Loss >10% (n = 11)	p	Normal Muscularity (n = 9)	Low Muscularity (n = 32)	p	Not Low Muscularity + >2% W/L (n = 24)	Low Muscularity + >2% W/L (n = 17)	p
Age	65±2	67±2	63±2	0.216	65±2	66±2	0.849	61±3	66±2	0.128	65±2	66±2	0.727
Sex (M:F)	30:11	19:4	11:7		23:7	7:4		4:5	26:6		19:5	11:6	
Pre Illness Weight (kg)	83±3	83±4	81±4	0.748	82±3	83±5	0.912	86±7	81±3	0.487	84±4	81±4	0.602
Weight Loss (%)	5±1	0±1	12±1	**0.000***	2±1	15±2	**0.000***	5±3	6±1	0.752	2±1	11±1	**0.000***
BMI	26±1	28±1	24±1	**0.016***	27±1	24±1	**0.048***	29±2	25±1	**0.050***	28±1	24±1	**0.013***
Body Fat (kg)	17±1	18±2	16±1	0.416	17±1	16±1	0.609	16±3	17±1	0.768	18±2	16±1	0.258
Fat Free Mass Index (FFMI) (kg/m^2)	16±0	16±1	15±1	0.057	16±1	15±1	0.143	17±1	15±0	**0.026***	17±1	14±0	**0.001***
CRP (mg/L)	14.5±5	16±8	12±5	0.667	13±6	18±8	0.670	15±9	14±6	0.966	10±4	20±10	0.301

Data (except gender split) are presented as mean (SEM).
* = cachexia group significantly different from the non-cachexia group, ($p<0.05$ by Student's t test).
Abbreviations – CRP = C - reactive protein, BMI = Body Mass Index.
doi:10.1371/journal.pone.0083618.t001

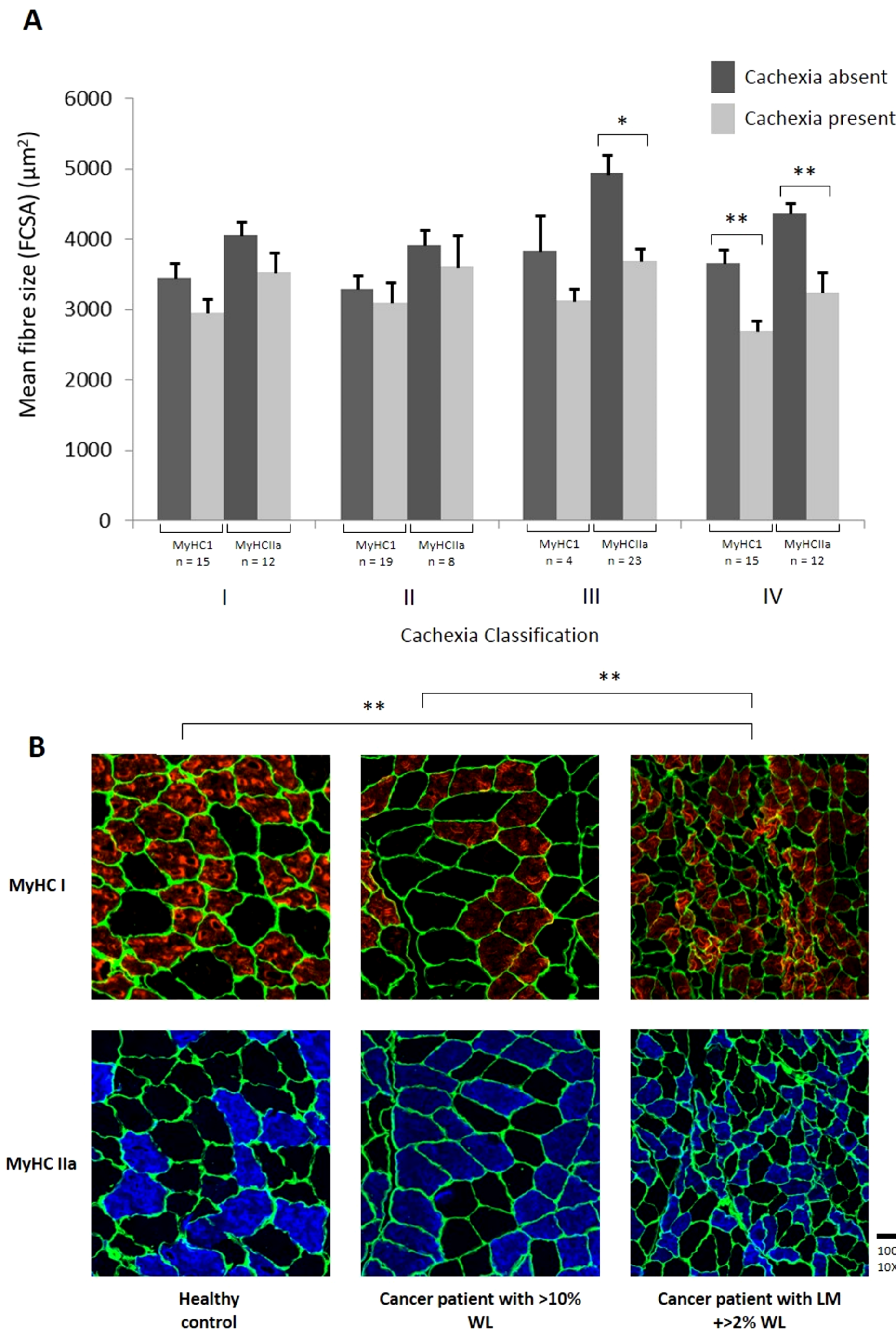

Figure 1. Fibre type cross sectional area (FCSA) according to different definitions of cachexia. (A) Mean (± SEM) fibre size for both MyHC1 and MyHCIIa. A comparison is made between patients with the proposed cachexia definition absent (dark grey) and those with the proposed cachexia definition present (light grey) for the four definitions set out in Methods (I–IV). (*, $P<0.05$ and **, $P<0.01$, by Student's t test). (B) Immunohistological sections of muscle for a healthy control, patient with weight loss alone (10.1%) (Group II), and patient with low muscularity and >2% weight loss (Group IV). Laminin is shown in green, MyHC1 shown in red, and MyHCIIa is shown in blue.
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– cachectic patients according to any of the diagnostic criteria (Table 3).

SMAD Signalling

In patients with >5% WL, SMAD3 protein levels were significantly increased when compared with non-cachectic patients (Figure 4). There were no significant differences in phospho-SMAD3/SMAD3 across any of the groups (Figure 4).

Inflammatory Pathways

Systemic inflammation was estimated using patients' serum CRP levels (Table 1). Patients were classified as having systemic inflammation if their CRP was ≥10 mg/L. There was no

Table 2. Myosin heavy chain fibre data of the patients involved in the study.

	All Patients (n = 27)	Weight Stable (n = 15)	Weight Loss >5% (n = 12)	p-Value	Weight Stable (n = 19)	Weight Loss >10% (n = 8)	p-Value	Normal Muscularity (n = 4)	Low Muscularity (n = 23)	p-Value	Not Low Muscularity + >2% W/L (n = 15)	Low Muscularity + >2% W/L (n = 12)	p-Value
Age	66±2	67±2	64±2	0.321	66±2	65±2	0.791	63±3	66±2	0.370	65±2	66±2	0.882
Sex (M:F)	24:3	15:0	9:3		18:1	6:2		4:0	20:3		15:0	9:3	
Pre Illness Weight (kg)	85±4	83±5	88±5	0.537	83±4	89±6	0.482	104±12	82±3	**0.018***	86±5	84±5	0.737
Weight Loss (%)	5±1	0±1	12±1	**0.000***	2±1	14±2	**0.000***	5±2	5±2	0.947	1±1	11±2	**0.000***
BMI at time of biopsy	26±1	27±2	25±1	0.381	29±1	25±1	0.454	33±3	25±1	**0.003***	28±1	24±1	**0.035***
Body Fat (kg)	25±1	18±2	18±1	0.980	18±1	17±1	0.848	22±3	17±1	0.103	19±2	16±1	0.255
Free Fat Mass (FFM) (kg)	48±2	49±2	46±2	0.217	48±2	46±2	0.459	59±3	46±1	**0.000***	51±2	44±2	**0.012***
Free Fat Mass Index (FFM) (kg/m^2)	27±0	16±1	15±1	0.303	16±1	15±1	0.511	19±1	15±0	**0.000***	17±1	14±0	**0.005***
Mean fibre size (FCSA) (μm^2)													
MyHC All Data	3588±172	3800±190	3324±297	0.167	3639±180	3468±412	0.514	4509±382	3408±172	**0.020***	4045±169	2979±234	**0.001***
Group MyHCI	3232±169	3446±241	2963±225	0.183	3287±206	3101±319	0.621	3832±616	3127±167	0.145	3653±229	2705±159	**0.004***
Group MyHCIIa	3831±195	4076±195	3524±361	0.167	3924±201	3611±478	0.482	4934±290	3698±193	**0.016***	4365±164	3238±297	**0.002***
Total fibre number per mm^2													
MyHC All Data	1228±147	1007±224	1504±187	0.152	1018±184	1726±252	0.119	937±179	1296±169	0.398	1226±227	1263±183	0.905
Group MyHCI	627±96	525±160	756±92	0.427	506±127	915±133	0.217	406±86	666±112	0.353	653±160	596±97	0.777
Group MyHCIIa	621±94	503±147	769±101	0.067	519±120	863±131	0.163	594±117	693±106	0.706	682±146	676±142	0.976
Fibre Type (%)													
MyHCI	48±3	49±4	41±3	0.128	48±3	38±5	0.079	43±3	46±3	0.691	48±4	42±4	0.230
MyHCIIa	55±2	52±3	59±2	0.107	54±3	58±3	0.388	64±3	53±2	0.099	55±3	55±3	0.978

Data (except gender split) are presented as mean (SEM).
* = cachexia group significantly different from the non-cachexia group, ($p<0.05$ by Student's t test).
Abbreviations – BMI = Body Mass Index, FCSA = Fibre Cross Sectional Area.
doi:10.1371/journal.pone.0083618.t002

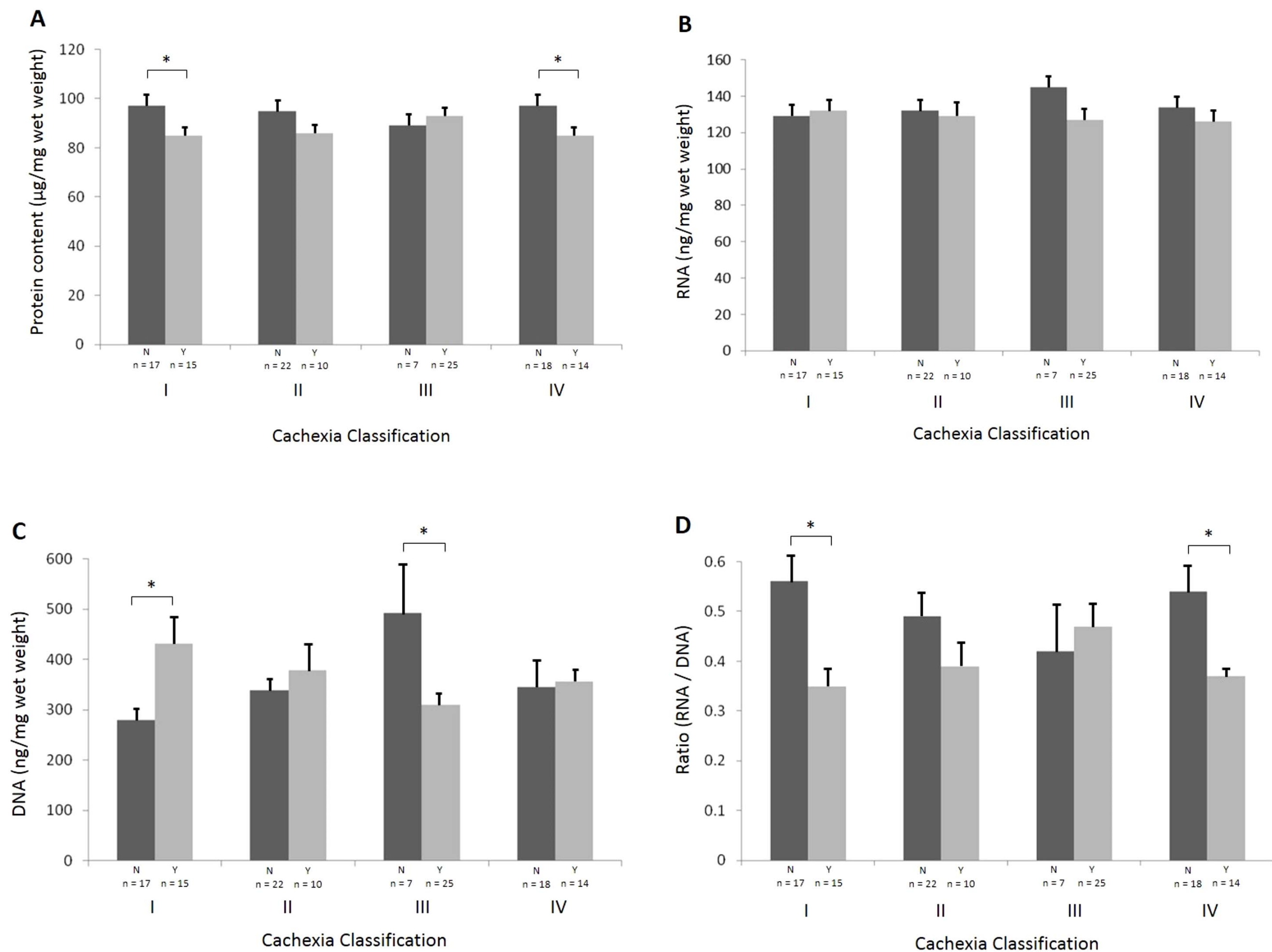

Figure 2. Variations in protein and nucleic acid content according to the different definitions of cancer cachexia. A comparison is made between patients with the proposed cachexia definition absent (dark grey) and those with the proposed cachexia definition present (light grey) for the four definitions set out in the methods (I–IV). (A) Mean (± SEM) wet weight protein content. (B) Mean (± SEM) RNA content. (C) Mean (± SEM) DNA content. (C) Mean (± SEM) RNA/DNA ratio. (*, P<0.05 by Student's t test).
doi:10.1371/journal.pone.0083618.g002

difference in the proportion of patients with or without systemic inflammation according to the definition of cachexia. Levels of phospho-NFκB and phospho-STAT3 were not significantly different in patients with or without cachexia (using any of the definitions: table 4) or with or without systemic inflammation (Figure 5).

Discussion

Fibre Size

The diagnostic criterion for cancer cachexia has long been based on weight loss alone [1] and can reflect loss in either fat or lean tissue compartments. Given that the key tissue loss in cancer cachexia is considered to be skeletal muscle, a recent consensus process suggested that the diagnostic criteria for cachexia should also take account of low baseline levels of muscularity [1]. In the present study, when patients were classified as cachectic or not according to ≥5% weight loss there was no significant difference in whole body muscularity (FFMI) or muscle fibre CSA. In contrast, when patients were classified according to low muscularity and ≥2% weight loss, FFMI was decreased and fibre cross sectional area was also significantly reduced (Figure 1). Such findings demonstrate that heterogeneity in relation to low muscularity and fibre atrophy may be reduced according to the clinical definition of cachexia. This finding may be important especially when considering inclusion criteria for clinical trials that aim to test the efficacy of drugs targeted at reversal of muscle wasting in cancer patients. The reduction in fibre size in all MyHC isoforms observed in the present study is consistent with previous animal [20] and human studies of cancer cachexia [4–7]. The rectus muscle of patients with oesophago-gastric cancer cachexia has been shown to lose all type MyHC content as well as undergo a reduction in fibre size [4]. Equally, in pancreatic cancer patients with cachexia, both type I and type II MyHC protein levels were decreased by 45% when compared with controls [6].

Fibre Type

In order to study differences in muscle fibre morphology and composition within the different cachexia categories, we performed immunohistochemical analysis of human muscle samples. For that, we first established and validated the staining methods for the myosin heavy chain antibodies specific for the different fibre types (I, IIa, and IIb). Staining for type I and IIa fibres resulted in strong specific staining specificity, however only weak

Table 3. Protein, DNA, and RNA content of the patients involved in the study.

	All Patients (n = 32)	Weight Stable (n = 17)	Weight Loss >5% (n = 15)	p-Value	Weight Stable (n = 22)	Weight Loss >10% (n = 10)	p-Value	Normal Muscularity (n = 7)	Low Muscularity (n = 25)	p-Value	Not Low Muscularity + >2% W/L (n = 18)	Low Muscularity + >2% W/L (n = 14)	p-Value
RNA (ng/mg wet weight)	131±6	129±8	132±9	0.859	132±7	129±13	0.847	145±9	127±7	0.220	134±8	126±10	0.520
DNA (ng/mg wet weight)	351±34	279±33	431±54	**0.019***	338±38	378±66	0.582	493±103	310±28	**0.020***	345±53	357±34	0.866
Ratio (RNA/DNA)	0.46±0.05	0.56±0.07	0.35±0.04	**0.013***	0.49±0.06	0.39±0.05	0.254	0.42±0.11	0.47±0.05	0.622	0.54±0.07	0.37±0.02	**0.050***
Protein content (μg/mg wet weight)	92±2.7	97±4	85±2	**0.015***	95±4	86±3	0.132	89±4	93±3	0.575	97±4	85±2	**0.035***
Protein content/RNA	0.77±0.05	0.83±0.09	0.71±0.07	0.273	0.79±0.07	0.75±0.09	0.742	0.63±0.05	0.82±0.07	0.154	0.78±0.08	0.76±0.07	0.827

Data (except gender split) are presented as mean (SEM).
* = cachexia group significantly different from the non-cachexia group, ($p<0.05$ by Student's t test).
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staining was observed against type IIb MyHC, this finding has also been reported elsewhere [19]. The predominant types of MyHC fibre in rectus abdominis muscle are I and IIa and only <8% of type IIb positive fibres have previously been described [21]. Of the adult skeletal isoforms, each are expressed to varying degrees in both mouse and human skeletal muscle. However, although MyHCIIb is highly expressed at both the messenger RNA (mRNA) and protein level in murine skeletal muscle, evidence to date suggests that this isoform is effectively only expressed at the mRNA level in a very small subset of specialised muscles in the adult human [22]. As mentioned above, MyHCIIb expression is typically associated with high forces of contraction combined with rapid contractile characteristics and it has been suggested that the contractile characteristics of MyHCIIb may be incompatible with the biomechanical constraints of larger muscles [23], which may account for the lack of specificity found in the rectus muscle of our patient population.

In cachexia there is conflicting evidence as to whether there is selective loss of fibre type. There was no evidence for selective loss of fibre type in the present study (Table 2). Evidence from animal models suggests that Type II fibres are targeted selectively [24], with relative preservation of type I fibres in fasting [25], exposure to glucocorticoids [26], sepsis [27] and in the gastrocnemius muscle of the C26 model of cancer cachexia [28,29]. Models of cardiac cachexia, however have suggested a trend to selective loss of type I fibres and an increase in type II fibre [30]. Furthermore, not all groups have demonstrated Type I and II fibre differences even in animals. Indeed in a recent study of the C26 cachectic mouse model, both glycolytic and oxidative fibres of (extensor digitorum longus) EDL muscle underwent wasting [20], whilst in a previous study using the same mouse model there was a significant increase in the amount of MyHCIIb and a significant decrease in the amount of type 1 MyHC in soleus muscle [31]. It is currently not entirely clear which type of fibres are affected in human cancer cachexia, however, in patients with oesophago-gastric cancer cachexia early loss of all MyHC isoforms has been reported [4].

The activity patterns of a muscle are also key in determining phenotype. If muscle cells are recruited infrequently they develop into fast/glycolytic units whereas if they are recruited more often, they form slow/oxidative units. In the C26 mouse model of cancer cachexia, there have been reports of switching of myosin isoforms in the soleus muscle of cachectic mice [31]. In pancreatic cancer patients with cachexia, no difference in the ratio of fast/slow myosin isoform was demonstrated compared with controls [6].

Muscle RNA, DNA, and Protein Content

In the present study, when compared with non-cachectic patients, muscle protein content was reduced significantly (approximately 13%) if patients were classified as cachectic by either >5% WL ($p = 0.015$) or LM + >2%WL ($p = 0.035$), and by 10% in patients with >10% WL. Protein content expressed in relation to wet weight of muscle has been shown to decrease progressively (in excess of 50%) in the gastrocnemius muscle of mice bearing the MAC-16 tumour [32]. This suggests that not only is there loss of fibre diameter, but that the quality of the fibre is altered with loss of either sarcoplasmic or myofibrillar protein. Such changes in fibre composition may contribute to the reduced muscle mechanical quality (force per unit cross-sectional area) observed in human cancer cachexia [33].

A reduction in both RNA content and activity in skeletal muscle has been attributed to a depression of protein synthesis in mice bearing the MAC16 tumour [32] . In the present study, RNA content was unaltered in cachectic patients (classified either with >5% WL or LM + >2% WL) compared with non-cachectic

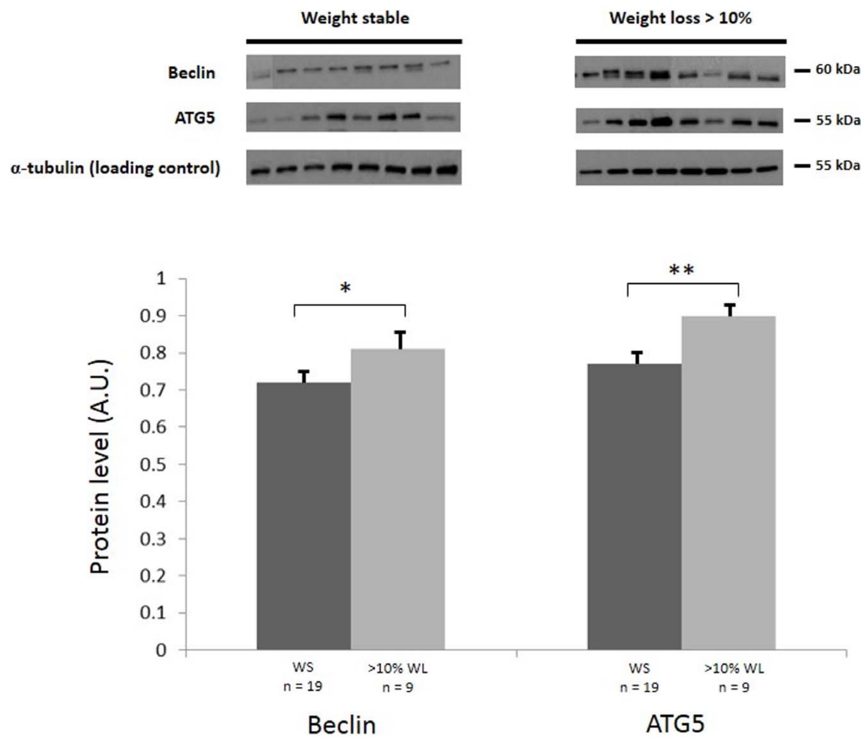

Figure 3. Skeletal muscle Beclin and ATG5 protein levels in patients with or without >10% weight loss (Group II). Western blot analysis with indicated antibodies, α-tubulin was used as a loading control. Graph shows the mean (± SEM) protein level represented in arbitrary units (A.U). (*, P<0.05 and **, P<0.01, by Student's t test).
doi:10.1371/journal.pone.0083618.g003

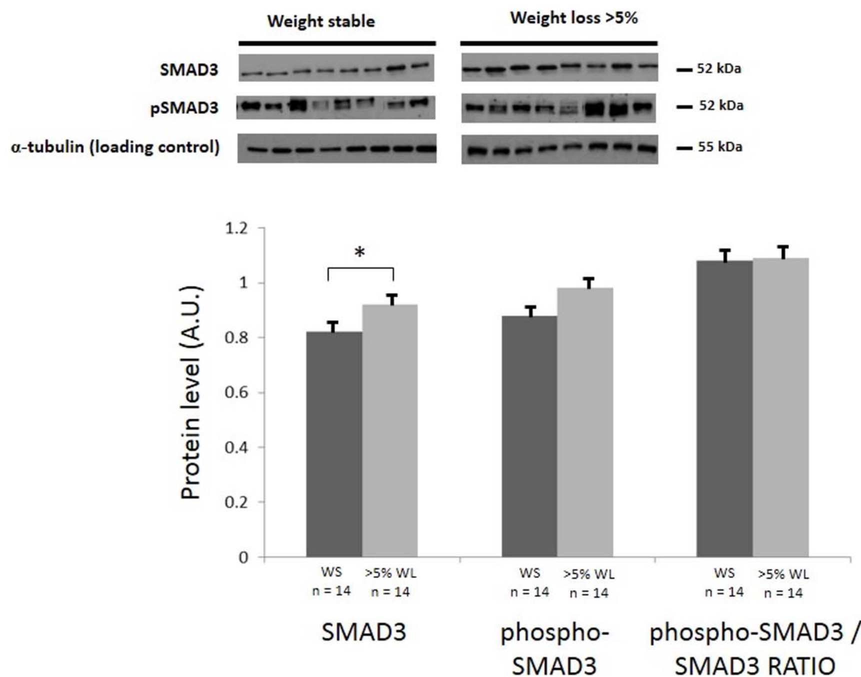

Figure 4. Total SMAD3, phospho-SMAD3 and ratio of phospho-SMAD3/SMAD3 in patients with or without >5% WL (Group I) levels. Western blot analysis with indicated antibodies, α-tubulin was used as a loading control. Graph shows the mean (± SEM) protein level represented in arbitrary units (A.U). (*, P<0.05 by Student's t test).
doi:10.1371/journal.pone.0083618.g004

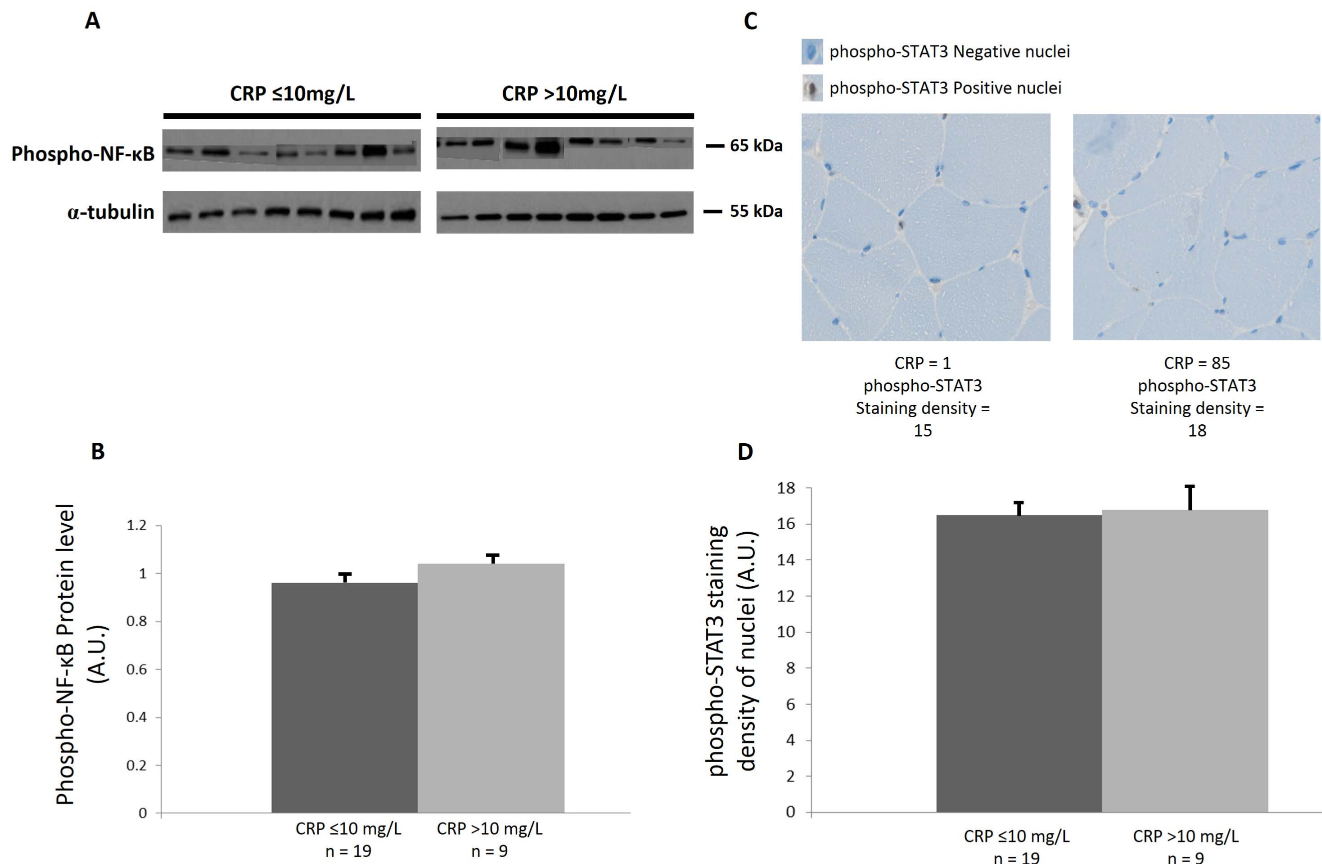

Figure 5. Inflammatory pathways in patients with (CRP >10 mg/L) and without (CRP ≤10 mg/L) systemic inflammation. (A) Western blot analysis in the presence or absence of systemic inflammation with indicated antibodies, α-tubulin was used as a loading control. (B) Graph shows the mean (± SEM) protein level of phospho-NF-κB, represented in arbitrary units (A.U). (C) Representative immunohistochemistry and nuclei count of phospho-STAT3 (area shown is representative of field) of a patient with or without systemic inflammation. (D) Graph shows the staining density of phospho-STAT3 nuclei (A.U.) (± SEM) in the presence or absence of systemic inflammation.
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patients. A reduction in the RNA content in the muscle of mice bearing the Ehrlich ascites tumour has also been reported, but this occurred later than the observed depression in the rate of protein synthesis [34]. Whether muscle protein synthesis is depressed in human cancer cachexia remains to be resolved [10].

In a murine model of cancer cachexia DNA content of the gastrocnemius muscle has been shown to remain relatively constant, despite the finding of a decrease in protein and RNA content [32]. The current study demonstrated DNA content was increased by >50% with >5% WL but decreased by 40% in patients with LM (Figure 2C). Because mature myofibre nuclei are thought to be mitotically inactive, increased DNA content in skeletal muscle cells suggests activation of satellite cells [35] or infiltration by other cell types such as inflammatory cells or adipocytes. In the LM group, the decrease in DNA may be due to pre-existing age-related sarcopenia or other causes of muscle atrophy (e.g. immobilisation) and may relate to muscle specific apoptosis and reduction in cell number in keeping with a reduction in muscle mass on CT scanning. The diametrically opposite changes in muscle DNA content dependent on whether patients are classified according to weight-loss or low muscularity again underpin the potential diverse mechanisms whereby older cancer patients may develop a low level of muscularity.

The issue of whether nuclear domain size is reduced in cancer cachexia remains to be resolved. In particular, whether apoptosis in skeletal muscle is increased in cancer cachexia and the degree to which DNA content is maintained or not via a compensatory increase in myonuclear number (possibly via satellite cell nuclei incorporation) is not known. Features of cachexia such as hypogonadism (resulting in low testosterone) or systemic inflammation (associated with high IL-6) could influence such regenerative capacity. In the current study RNA/DNA was altered in the cachectic patients (independent of definition) compared with the non- cachectic patients. This may be due to the interplay of the mechanisms described above.

Mechanisms

Skeletal muscle atrophy may occur as a result of decreased synthesis, increased degradation or both [36]. In mice bearing the MAC-16 adenocarcinoma, muscle loss is due to the combination of reduced synthesis and increased degradation [37]. Similarly Samuels et al demonstrated reduced protein synthesis and increased degradation in skeletal muscle co-incident with the onset of cachexia in mice implanted with the C26 murine model [38].

Degradation Pathways. The majority of signalling pathways contributing to muscle atrophy in pre-clinical models are mediated through activation of the ubiquitin-proteasome proteolytic pathway (UPP) [39]. The muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1 are up regulated in animal models of acute

Table 4. Western blots for protein markers of cellular signalling.

	Weight Stable (n = 14)	Weight Loss >5% (n = 14)	p-Value	Weight Stable (n = 19)	Weight Loss >10% (n = 9)	p-Value	Normal Muscularity (n = 8)	Low Muscularity (n = 20)	p-Value	Not Low Muscularity + >2% W/L (n = 17)	Low Muscularity + >2% W/L (n = 11)	p-Value
Beclin (A.U)	0.73±0.03	0.77±0.04	0.452	0.72±0.02	0.81±0.05	**0.050***	0.70±0.03	0.77±0.03	0.232	0.72±0.03	0.79±0.04	0.153
ATG5 (A.U)	0.80±0.03	0.83±0.04	0.614	0.77±0.03	0.9±0.04	**0.013***	0.75±0.05	0.84±0.03	0.137	0.81±0.03	0.82±0.05	0.865
ATG12 (A.U)	0.86±0.04	0.87±0.04	0.898	0.84±0.03	0.93±0.05	0.090	0.83±0.05	0.89±0.03	0.313	0.86±0.03	0.88±0.04	0.720
ATG7 (A.U)	0.89±0.05	0.87±0.05	0.741	0.85±0.04	0.96±0.07	0.136	0.85±0.08	0.89±0.04	0.617	0.89±0.04	0.86±0.06	0.682
phospho-SMAD3 (A.U)	0.88±0.04	0.98±0.04	0.116	0.91±0.04	0.97±0.04	0.343	0.87±0.07	0.95±0.03	0.171	0.90±0.04	0.98±0.04	0.191
SMAD3 (A.U)	0.82±0.03	0.92±0.03	**0.022***	0.86±0.02	0.89±0.04	0.497	0.88±0.03	0.87±0.03	0.816	0.85±0.02	0.89±0.04	0.348
phospho-SMAD3/SMAD3 (A.U)	1.08±0.06	1.08±0.05	0.972	1.07±0.05	1.11±0.07	0.630	0.98±0.05	1.12±0.05	0.107	1.06±0.05	1.12±0.06	0.467
phospho-NF-κB (A.U)	0.97±0.03	1.00±0.04	0.608	0.96±0.02	1.03±0.05	0.116	0.94±0.03	1.00±0.03	0.258	0.97±0.02	1.01±0.04	0.347
phospho-STAT3 staining density of nuclei (A.U.)	17.9±1.34	15.5±0.62	0.105	17.4±1.03	15.1±0.74	0.170	16.3±0.95	16.7±1.00	0.817	17.2±1.10	15.6±0.80	0.311

Data are presented as mean (SEM). Immunohistology data is shown for phospho-STAT3 and is recorded as staining density of nuclei (A.U.).
* = cachexia group significantly different from the non-cachexia group, ($p<0.05$ by Student's t test).
Abbreviations – A.U = arbitrary units.
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atrophy [40,41], and MuRF1 selectively targets the myofibrillar protein myosin heavy chain resulting in muscle wasting [8]. However, the role of the E3 ligases in human cachexia is less well defined. In the current study we chose not to measure directly these pathways as results from our previous investigation on a similar cohort of patients found no up regulation using a transcriptomics approach [42], this has also been validated recently in a separate cohort of patients with gastric cancer [43]. In the present study autophagy proteins (ATG) 5, 7, 12, and beclin 1 were studied. These proteins are necessary for autophagy due to their role in autophagosome elongation [44]. When patients were classified according to >10% WL, Beclin and ATG5 protein levels were significantly increased in cachectic patients when compared with non-cachectic patients. In a previous study in a similar cohort of patients, the autophagy related genes GABRAPL1 and BNIP3 were increased in rectus muscle biopsies from cachectic versus non-cachectic patients [42]. In normal muscle, low-protein diets up-regulate autophagy that leads to the loss of muscle mass at least partially through lysosomal degradation [45]. Intriguingly, under other circumstances decreased autophagy can also lead to muscle atrophy.

Systemic Inflammation. Systemic inflammation is thought to be a major mediator of cancer cachexia [10]. However, the relationship between inflammation in the systemic compartment versus muscle and the relationship of either to muscle loss in humans is not clear. In the systemic compartment, Il-6 is thought to be a major mediator and may signal within target organs via STAT-3. Alternatively, both IL-1 and TNF alpha may signal via NF-kB. NF-kB regulation of muscle atrophy is predominantly executed by promoting proteasome-mediated degradation [46]. Activation of NF-kB has been detected in both physiological and pathological atrophic conditions such as denervation, unloading, aging, cancer, sepsis, diabetes, and such atrophy can be reversed by pharmacologic or genetic NF-kB inhibition [47]. In the present study although there was evidence for systemic inflammation in a proportion of patients, no significant difference was found in the levels of phospho-NFkB or phospho-STAT3 across any of the definitions of cachexia or in those with or without evidence of systemic inflammation. It is possible that inflammatory mediators have their main effects on muscle atrophy via central mechanisms mediated via the CNS [48].

SMAD3. It has been suggested that binding of myostatin to the ActRIIB receptor results in the phosphorylation of two serine residues of SMAD2 or SMAD3. This leads to the assembly of SMAD2/3 with SMAD4 to the heterodimer that is able to translocate to the nucleus and activate transcription of target genes [49]. One of the known downstream targets of SMAD signalling is MyoD, a transcriptional factor that is involved in skeletal muscle development and takes part in the repair of damaged skeletal muscle [50]. Moreover, SMAD signalling targets other genes such as myf5 and myogenin, known to be important for myogenesis [51]. Myostatin is upregulated in cachexia and in states of muscle paralysis [52]. Myostatin/ActRIIB activates SMAD2/3 signalling and importantly SMAD2/3 inhibition completely desensitises ActRIIB-induced muscle atrophy [13]. Inhibition of myostatin by a dominant negative ActRIIB promotes muscle hypertrophy independent of muscle satellite cell recruitment consistent with a direct signalling effect on muscle catabolism [13]. When patients were classified as cachectic according to >5% WL, SMAD3 protein levels were significantly increased in cachectic patients when compared with non-cachectic patients. Equally there was a similar (but not significant) increase in phospho-SMAD3 associated with >5% weight loss. It is not known whether such increased

protein levels indicate increased pathway activity independent of any alteration in the ratio of phospho-SMAD3/SMAD3.

Limitations of Study

It is important to appreciate that the majority of patients in the present series will have had some degree of age-related sarcopenia, that this will necessarily co-exist with any cancer specific loss of skeletal muscle mass and that the diagnostic criteria used in the present study will not necessarily separate one from the other. The current study was not longitudinal and it was therefore not possible to document active muscle loss. It is also important to recognise that when patients were divided into different diagnostic categories the sample size in individual categories may have limited the ability to detect a statistical difference or not. This was an exploratory study and provides the basis for a larger study with adequate statistical power for definitive analysis.

Conclusions

In the present study, when the diagnostic criteria for cachexia included both a measure of low muscularity and weight loss, muscle fibre size, protein content and RNA/DNA content were all reduced. Such consistent findings were not observed when cachexia was diagnosed based on weight-loss or low muscularity alone. Whereas fibre type is not targeted selectively, muscle fibre size, biochemical composition and pathway phenotype can vary according to whether the criteria for cachexia include both a measure of low muscularity and weight loss. Such findings suggest that current diagnostic criteria identify groups of patients with different skeletal muscle phenotypes. Identification of a more homogeneous patient cohort for musculo-centric intervention trials may require use of combined criteria.

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Author Contributions

Conceived and designed the experiments: JAR RR DJG CJ KCHF. Performed the experiments: NJ SH NAS MD SD WF CL. Analyzed the data: NJ SH CJ KCHF. Contributed reagents/materials/analysis tools: MD SD WF CL. Wrote the paper: NJ SH NAS JAR RR DJG CJ KCHF. N/A.

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Review

Muscle wasting in cancer☆

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ABSTRACT

Skeletal muscle loss appears to be the most significant clinical event in cancer cachexia and is associated with a poor outcome. With regard to such muscle loss, despite extensive study in a range of models, there is ongoing debate as to whether a reduction in protein synthesis, an increase in degradation or a combination of both is the more relevant. Each model differs in terms of key mediators and the pathways activated in skeletal muscle. Certain models do suggest that decreased synthesis accompanied by enhanced protein degradation via the ubiquitin proteasome pathway (UPP) is important. Murine models tend to involve rapid development of cachexia and may represent more acute muscle atrophy rather than the chronic wasting observed in humans. There is a paucity of human data both at a basic descriptive level and at a molecular/mechanism level. Progress in treating the human form of cancer cachexia can only move forwards through carefully designed large randomised controlled clinical trials of specific therapies with validated biomarkers of relevance to underlying mechanisms.

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Abbreviations: ACTRIIB, activin receptor type-2B; APC, adenomatosis polyposis coli; APPR, acute phase protein response; ARC, arcuate nucleus; ATP, adenosine-5′-triphosphate; C-26, colon-26 adenocarcinoma mouse model; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CRPc, reactive protein; CSA, cross sectional area; DGC, dystrophin glycoprotein complex; DM, diabetes mellitus; DNA, deoxyribonucleic acid; EDL, extensor digitorum longus; EIF3F, eukaryotic translation initiation factor 3 subunit F; F-bxo40, F-box protein 40; FCSA, fibre cross sectional area; FOXO, forkhead box class O transcription factor; ICU, intensive care unit; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; IRS, insulin receptor substrate; JAK, Janus associated kinase; LBM, lean body mass; LLC, Lewis lung carcinoma; MA, megestrol acetate; mAb, monoclonal antibody; MAC16, murine adenocarcinoma 16 mouse model; MAFbx, muscle-specific F-box (also known as atrogin-1); MAPK, mitogen activated kinase; MCR, melanocortin receptor; MRI, magnetic resonance imaging; mRNA, messenger ribonucleic acid; MSH, melanocyte-stimulating hormone; mTOR, mammalian target of rapamycin; MURF-1, muscle-specific RING finger-1; MyHC, myosin heavy chain; NFκB, nuclear factor-κβ; NPY, neuropeptide Y; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cell; PI3K, phosphatidylinositol 3-kinase; POMC, pro-opiomelanocortin; QoL, quality of life; RNA, ribonucleic acid; STAT, signal transducer and activator of transcription; TA, tibialis anterior; TAM, tumour-associated macrophage; TGF, transforming growth factor; T_h, T helper; TNF, tumour necrosis factor; TWEAK, tumour necrosis factor-like weak inducer of apoptosis; Ub, ubiquitin; UPP, ubiquitin-proteasome pathway.

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1. Introduction

Weight loss in cancer patients is associated with excess morbidity and mortality (Rennie, 2009; Brandt and Pedersen, 2010). Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life and duration of survival. Cancer cachexia has recently been defined 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 (Fearon et al., 2011). Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients' function and quality of life and is clearly associated with a poor outcome (Tan et al., 2009; Fearon et al., 2011; Miller et al., 2012).

2. Heterogeneity

Heterogeneity both in clinical and animal models is one of the key issues that has impaired research into cancer cachexia. Cachexia is not a single phenomenon but evolves through a spectrum of pre cachexia, cachexia, and refractory cachexia (Fearon et al., 2011). The incidence and severity of cachexia can vary according to tumour type, site, and mass (Dewys et al., 1980). Equally, the contribution of reduced food intake versus abnormal metabolism can vary considerably (Knox et al., 1983). The components of such abnormal metabolism also vary, and within the same model/individual can evolve with time such that hypermetabolism (Zylicz et al., 1990) or activation of proteolytic pathways (Khal et al., 2005a,b) occurs during the early phase of cachexia but not during a more advanced phase. In humans the cause of such heterogeneity relates not only to the clinical status of the patient and specific effects of the tumour (e.g. causing bowel obstruction, tissue destruction or concomitant infection) but also to co-existing morbidities (e.g. heart failure, chronic renal failure, or chronic obstructive pulmonary disease), age related sarcopenia and the possibility of a genetic predisposition to develop cachexia (Fig. 1).

There are numerous animal models of cancer cachexia. A recent review concluded that for research into cancer cachexia where there is little evidence of systemic inflammation, the murine adenocarcinoma 16 mouse (MAC-16) and XK1 murine tumour models are useful (Bennani-Baiti and Walsh, 2011). Weight loss in mice bearing the MAC-16 tumour appears to be independent of reduced food intake or inflammation (Bennani-Baiti and Walsh, 2011). In contrast the XK1 model demonstrates reduced food intake. All other models induce a host inflammatory response. In the Walker 256 and MCG – 101 models tumour growth is extremely rapid resulting in a tumour mass exceeding 10% of host body weight in a matter of days (Bennani-Baiti and Walsh, 2011). This highlights the problems with the translational value of relatively acute onset cancer cachexia seen in murine models compared with humans where a cancer may spread over a period of months or years (with similar time scale for the evolution of cachexia). One of the most popular models at present is a particular clone of the colon-26 adenocarcinoma (C26) tumour in mice. This is thought to be a mainly IL-6 dependant model of cachexia (Strassmann et al., 1992). Whether, in fact, this can be considered as representative of the majority of cancer patients remains highly speculative.

3. Skeletal muscle morphology in cancer associated myopenia: murine models

Skeletal muscle is composed of muscle fibres which are classified according to their speed of contraction and predominant type of energy metabolism. Muscle fibres can be classified as type I, slow-twitch and type II, fast-twitch fibres based on their predominant myosin heavy chain (MyHC) isoform content. Generally, type I and type IIa fibres utilise oxidative phosphorylation as their energy source, whereas type II*x* and IIb fibres harness anaerobic metabolism to generate ATP (Schiaffino and Reggiani, 1996; Berchtold et al., 2000). Both the percentage and structural morphology of the fibre type will determine the phenotypic capacity and functional performance of any given muscle. Environmental factors in both health and disease have a direct impact on muscles leading to changes in fibre type and morphology which lead to changes in muscle functionality; such processes include ageing, exercise, diabetes, disuse atrophy, chronic heart failure, and cachexia (Lipkin et al., 1988; Mancini et al., 1989; Sullivan et al., 1990; Drexler et al., 1992; Belardinelli et al., 1995; Short and Nair, 2001; Basu et al., 2002; Marx et al., 2002; Short et al., 2004; Schmitt et al., 2007; Weber et al., 2007; Eley et al., 2008; Harber et al., 2009; Weber et al., 2009). The change, preservation or loss of fibres may influence clinical symptoms and there is some evidence that fibre

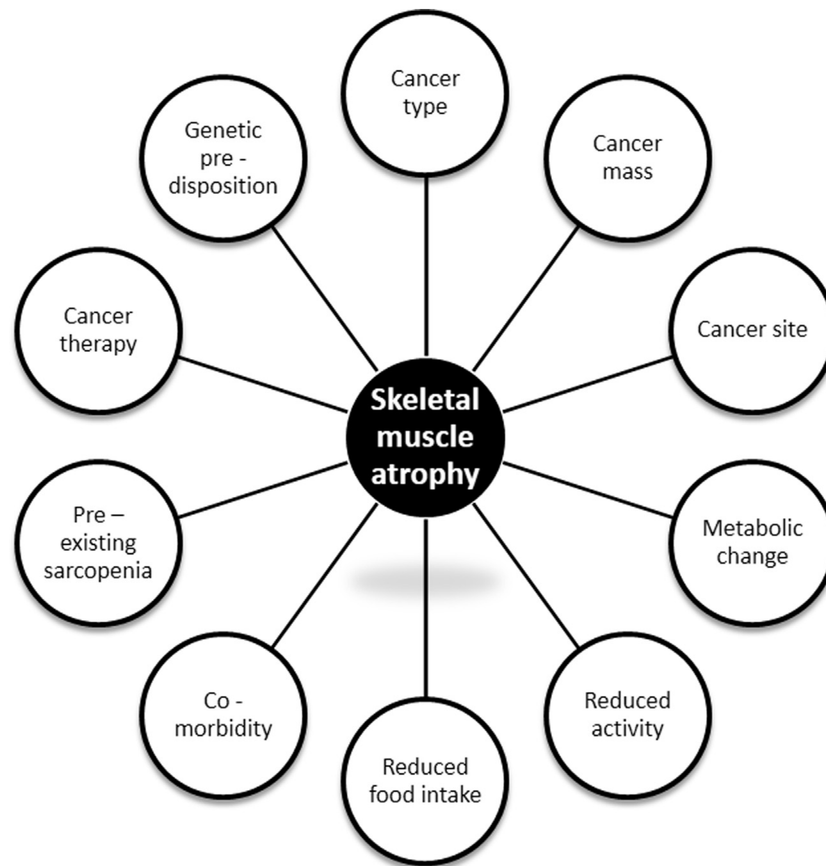

Fig. 1. Heterogeneity in cancer cachexia.

type is targeted selectively in cancer cachexia (Clarke et al., 2007; Cohen et al., 2009). Ongoing loss of protein in muscle tissue leads to muscle fibre shrinkage and a reduction in CSA. This loss of CSA leads to the main muscle groups in the body undergoing changes in fibre composition which leads to a marked loss of aerobic capacity (VO^2max) in healthy subjects as well as cancer patients (Ferretti et al., 1997; Proctor and Joyner, 1997; Bassett and Howley, 2000; Pette and Staron, 2001; Weber et al., 2007).

3.1. Fibre size and type in cancer associated myopenia

The model that has been most frequently studied is the C26 tumour grown subcutaneously in mice (Table 1). The predominant muscles studied include the gastrocnemius and soleus from the lower limb. Unlike humans, no model uses old, co-morbid mice undergoing concomitant anticancer therapy! A common finding is a decrease in fibre size. With regard to fibre type, evidence from animal models suggests that type II fibres are targeted selectively (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 in the gastrocnemius muscle of the C26 model of cancer cachexia (Acharyya et al., 2004). Not all studies have demonstrated type 1 and 2 fibre differences. Indeed, in a recent exposition of the C26 cachectic mouse model, both glycolytic and oxidative fibres of (extensor digitorum longus) EDL muscle underwent wasting (Aulino et al., 2010), whilst in a previous study using the same mouse model there was a significant increase in the amount of type 2b MyHC and a significant decrease in the amount of type 1 MyHC in soleus muscle (Diffee et al., 2002).

The activity patterns of muscle are also key in determining phenotype. If muscle cells are recruited infrequently, they develop into fast/glycolytic units whereas if they are recruited more often, they form slow/oxidative units. In the C26 mouse model of cancer cachexia, there have been reports of switching of myosin isoforms in the soleus muscle of cachectic mice (Diffee et al., 2002).

3.2. Fibre RNA, DNA, and protein content in cancer associated myopenia

A reduction in total RNA content has been associated with a depression in protein synthesis in mice bearing the human hypernephroma XK1 (Emery et al., 1984) and in mice bearing the MAC-16 tumour (Bhogal et al., 2006). Timing of sampling may be important in the results obtained from such muscle biopsies. A reduction in the RNA content in the muscle of mice bearing the Ehrlich ascites tumour has also been reported, but this occurred later than the onset of depressed protein synthesis (Lopes et al., 1989).

Protein content expressed in relation to wet weight of muscle has been shown to decrease progressively (in excess of 50%) in the gastrocnemius muscle of mice bearing the MAC-16 tumour (Bhogal et al., 2006). Similar changes have been documented in the Yoshida ascites hepatoma A130 cachexia model (Tessitore et al., 1993a,b). This suggests that not only is there loss of fibre diameter, but that the quality of the fibre is altered with loss of either sarcoplasmic or myofibrillar protein. DNA content of the gastrocnemius muscle has been shown to remain relatively constant, despite the finding of a decrease in protein and RNA content (Bhogal et al., 2006). Clearly not all DNA in a muscle can be ascribed to myonuclei. However, these findings are consistent with the trend towards fibre

Table 1
Experimental animal data on myosin composition of muscles in cancer cachexia.

Authors	Model	Muscle group	Myosin fibre type	Fibre size (FCSA) (μm^2)	Fibre percentage (%)
Aulino et al. (2010)	Mouse C26 colon	EDL	1	↓	↓
			2	↓	↓
Acharyya et al. (2005)	Mouse C26 colon	TA/gastrocnemius	1	No change	No change
			2	↓	↓
Diffee et al. (2002)	Mouse C26 colon	Soleus	1		↓
			2a		No change
			2b		↑

cytoplasmic atrophy without a decrease in myonuclear number observed in mature mice that undergo long term denervation injury (Wada et al., 2002). The issue of whether nuclear domain size is reduced in cancer cachexia remains to be resolved. In particular, whether apoptosis in skeletal muscle is increased in cancer cachexia (van Royen et al., 2000) and the degree to which DNA content is maintained or not via a compensatory increase in myonuclear number (possibly via satellite cell nuclei incorporation) is not known. Features of cachexia such as hypogonadism (resulting in low testosterone) or systemic inflammation (associated with high IL-6) could influence such regenerative capacity.

3.3. Dystrophin glycoprotein complex (DGC)

It is also important to recognise that in cancer cachexia there may be changes in the muscle cytoskeleton as well as in the myofibrillar contractile apparatus. In recent years, it has been demonstrated that alterations in the muscular dystrophy-associated DGC also occur in skeletal muscle (Acharyya et al., 2005). Using both the C26 and Lewis lung carcinoma (LLC) murine cancer models, it was shown that muscles from tumour-bearing mice exhibited membrane abnormalities, accompanied by reduced dystrophin (core DGC member) expression and increased glycosylation of DGC proteins (Acharyya et al., 2005). The DGC is thought to play a key role in maintaining muscle integrity during contraction cycles and clearly loss of this function may exacerbate overall damage to the muscle.

4. Mediators of cancer associated myopenia: murine models

4.1. Neural control

Muscle mass is clearly sensitive to food intake. The pathogenesis of cancer anorexia is multifactorial and reflects the complexity of the mechanisms controlling energy homeostasis under physiological conditions. The main molecular mechanisms regulating the cancer anorexia-cachexia syndrome include alterations in brain neurochemistry. In particular, the hypothalamic melanocortin system appears not to respond appropriately to peripheral inputs, and its activity is diverted largely towards the promotion of catabolic stimuli promoting metabolism of carbohydrates, lipids, and proteins in peripheral tissues leading to insulin resistance, increased lipolysis, and accelerated muscle proteolysis (Tisdale, 2002) (Fig. 2). Pro-inflammatory cytokines (TNFα and IL-1β) and hypothalamic serotonergic neurons have been implicated in the dysfunction of the hypothalamic melanocortin system (Inui, 1999). Two peptide systems in particular appear to be strongly influential in the control of feeding behaviour: these are the orexigenic neuropeptide Y (NPY), and the anorexigenic (pro-opiomelanocortin) POMC systems (Plata-Salaman, 2000). Many of these mediators exert their effects through changes in the NPY/POMC systems. Both originate in the hypothalamic arcuate nucleus (ARC) and extend projections widely over the brain (Plata-Salaman, 2000). They have been shown to be linked intricately with each other and to operate in parallel. POMC neurons are the source of the potent melanocortin neuropeptides, such as α-melanocyte-stimulating hormone (MSH), which, via interaction with the central MC3/4 – receptors, induce an anorectic state. The role of cytokines in cancer anorexia may be affected through influence on both the NPY and POMC systems. Hypothalamic IL-1 mRNA has been shown to be significantly increased in methylcholanthrene-induced sarcoma bearing rats. Levels of IL-1 in cerebrospinal fluid of the same rats are also increased and inversely correlate inversely with energy intake (Opara et al., 1995). However, injection of an IL-1 receptor antagonist ameliorates anorexia in the same model (Laviano et al., 2000). In another experimental animal system, injection of IL-1β into the hypothalamus within hours causes significant change in gene expression in skeletal muscle including up regulation of the UPP. This clearly demonstrates the potential for neural control of muscle protein synthesis and degradation (Braun et al., 2011). This mechanism has not been fully explored in cancer cachexia.

4.2. Pro-inflammatory mediators

Mediators regulating skeletal muscle atrophy in cachexia are thought to derive from immune or tumour cells, or the targeted tissues undergoing wasting (both adipose tissue and skeletal muscle). Inflammation is thought to be of paramount importance. Cancer cell rely on production of pro-inflammatory mediators for growth, protection from apoptosis, and promotion of angiogenesis/metastasis. The tumour may consequently initiate a cytokine cascade that has multiple, direct, and distant effects including the initiation of skeletal muscle protein degradation.

CHO cells transfected with the human TNF-α gene induced cachexia when implanted into nude mice (Oliff et al., 1987). A similar finding was observed with CHO cells constitutively producing IFN-γ (Argiles et al., 2003). An anti-IFN-γ monoclonal antibody was able to reverse the wasting syndrome associated with the murine Lewis lung carcinoma (Matthys et al., 1991). TNF-α and IFN-γ work co-operatively to down-regulate transcription of the MyHC gene in vitro and in vivo, but not other core myofibrillar proteins (Acharyya et al., 2004). Furthermore, MyHC protein expression was decreased in a specific fashion in the C26 model (Acharyya et al., 2004), a largely IL-6 dependent model (Strassmann et al., 1993). TNFα has been shown in vitro to inhibit both adipocyte and skeletal myocyte differentiation (Guttridge et al., 2000; Ruan et al., 2002). It also plays a role in insulin resistance attenuating the insulin signalling pathway (Hotamisligil, 1999). The presence of TNFα promoted atrophy in cultured myotubes, resulting from the induction of E3 ligase genes that mediate the breakdown of myofibrillar proteins by the UPP (Li et al., 2005; Frost et al., 2007; Moylan et al., 2008; Sishi and Engelbrecht, 2011). TWEAK, another important cytokine with structural properties similar to TNFα, can induce a cachectic phenotype in part by the induction of the E3 ligase MuRF1, and the subsequent degradation of MyHC at the thick filament of the sarcomere (Dogra et al., 2007; Mittal et al., 2010). A variety of tumours have been shown to secrete IL-6 and this can

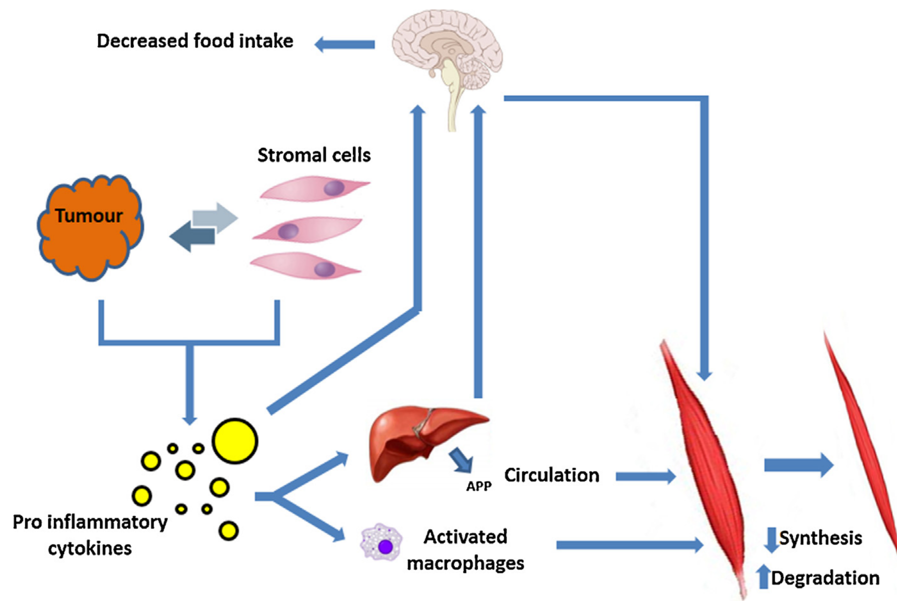

Fig. 2. Integrative physiology of cancer cachexia. The cancer is able to activate pro inflammatory, acute phase, and neuro – endocrine responses. The consequences are reduced food intake, decreased protein synthesis and increased degradation.

be amplified by host-derived proinflammatory cytokines (e.g. IL-1). Experiments regulating function in tumour bearing mice also support the requirement of IL-6 in cachexia (Black et al., 1991; Strassmann et al., 1992, 1993). In mice with a mutation in the APC tumour suppressor gene, elevated circulating IL-6 levels are associated with the presence of muscle wasting (Baltgalvis et al., 2008). Knockout of IL-6 prevents loss of muscle weight and epididymal fat, and reduces intestinal polyp number, implying the existence of an IL-6 cytokine amplification loop between host and tumour cells.

Other cytokines may be potential repressors of cachexia. For example, IL-4, IL-10 and IL-13 all demonstrate anti-inflammatory, and hence potentially anti-cachectic activity (Argiles and Lopez-Soriano, 1999). In the C26 mouse model, IL-10 gene transfer reduced cachexia and prolonged survival (Fujiki et al., 1997). Other cytokines (e.g. IL-15) may have potential 'antioxidant' properties that can counter the excess levels of reactive oxygen and nitrogen species (caused by the inefficiency of host antioxidant enzymes) that have been proposed as mediators of muscle atrophy (Barreiro et al., 2005). IL-15 was capable of inhibiting skeletal muscle wasting in the Yoshida AH-130 rat ascites hepatoma model by decreasing muscle protein degradation rates (Carbo et al., 2000). Overexpression of IL-15 in cultured myotubes induced a hypertrophic morphology and increased myofibrillar protein accumulation in co-cultured cells (Quinn et al., 2002).

4.3. *Catabolic mediators*

The interplay of anabolic and catabolic hormones has a distinct role in the development of skeletal muscle wasting. The catabolic hormones myostatin and activin are two members of the TGF-β superfamily that play an important role in growth and are thought to be responsible for the development of cachexia in mice lacking the hormone inhibin (Elliott et al., 2012). In mice, both molecules bind to activin receptor type-2B (ActRIIB), a receptor in muscle, to initiate a signalling cascade leading to increased expression of MuRF1 and MAFbx/Atrogin-1 and subsequent degradation of myofibrillar proteins (Glass, 2010a,b). In mice bearing the C26 tumour, administration of a soluble ACTRIIB decoy receptor resulted not only in reversal of muscle wasting but an increase in survival (Zhou et al., 2010).

4.4. *Anabolic mediators*

The anabolic hormone IGF1 has been implicated in the development of cancer cachexia in murine models. In the rat AH-130 hepatoma ascites model of cancer cachexia, muscle expression of IGF-1 mRNA decreased progressively whereas IGF-1 receptor and insulin receptor mRNA levels increased compared with controls (Costelli et al., 2006). Furthermore, circulating levels of IGF-1 and insulin were reduced. However, the exact mechanism of IGF-1 down-regulation and any role in muscle wasting in this scenario is unclear, as administration of exogenous IGF-1 to tumour-bearing rats did not prevent cachexia (Costelli et al., 2006), and other studies have shown, at least in experimental animal models of cancer cachexia, the IGF-1 signal transduction pathway is not down-regulated (Penna et al., 2010; White et al., 2011, 2013).

5. Mechanisms of cancer associated myopenia: murine models

5.1. *Protein degradation pathways*

Skeletal muscle atrophy may occur as a result of decreased synthesis, increased degradation or both (Glass, 2010a,b). In cancer cachexia it is evident that there is a complex interplay between synthesis and degradation. In mice bearing the MAC-16 adenocarcinoma, weight loss was accompanied by loss of whole body nitrogen in proportion to the overall loss of body mass. Using L-[4-3H]phenylalanine to label proteins in the gastrocnemius muscle to determine rates of synthesis and degradation, a significant depression (60%) in protein synthesis occurred in animals with a weight loss between 15% and 30%. This was accompanied by an increase in protein degradation, which increased with increasing weight loss between 15% and 30% (Smith and Tisdale, 1993). Three major pathways that may contribute to such increased protein degradation include the UPP, autophagy/lysosomal pathway, and the calcium dependant enzymes (e.g. calpains) (Tisdale, 2009).

5.1.1. *Ubiquitin proteasome pathway*

Various factors including malnutrition, impaired physical activity and the production of catabolic molecules (e.g.

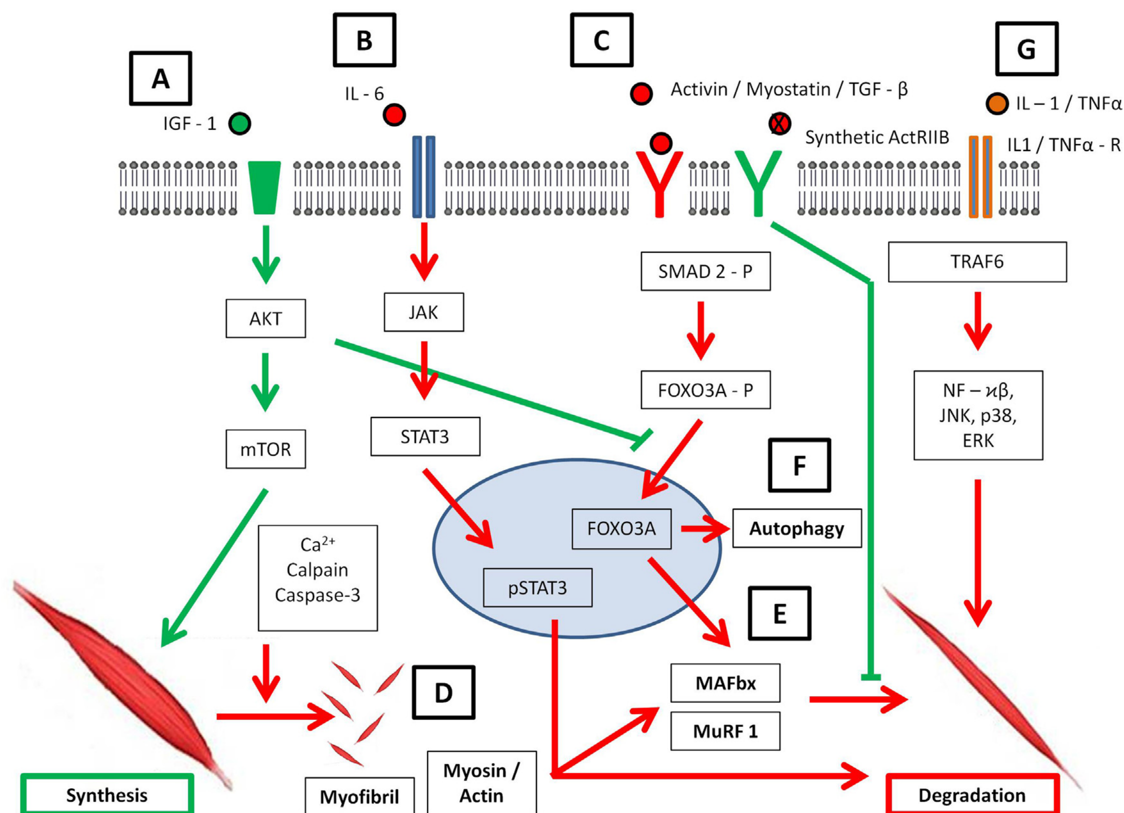

Fig. 3. Muscle protein kinetics in cancer cachexia. Regulation of muscle synthesis via IGF – 1 induced hypertrophy (A). Myokine activated JAK/STAT pathway involved in the acute phase response and muscle degradation (B). TGF-β induced SMAD pathway, activated by myostatin and activin (C). Calcium mediated mechanisms of muscle breakdown (D). Muscle breakdown mediated by UPP, leading to activation of the E3 Ubiquitin ligases MuRF1 and MAFbx (E). Activation and transcription of autophagy genes (F). NF–χβ signalling (G).

glucocorticoids and/or cytokines) up regulate rates of muscle proteolysis (Ventadour and Attaix, 2006). A common end point in pathways implicated for increased protein breakdown involves two muscle-specific E3 ubiquitin protein ligases, which target specifically a limited number of proteins for breakdown by the Proteasome. These E3 ligases are called MAFbx/Atrogin-1 and MuRF1 (Fig. 3).

5.1.1.1. Pro inflammatory cytokine activation of the UPP. TNFα, TWEAK or IL-1 signal to activate the NF–χβ pathway. NF–χβ influences a variety of pathways including apoptosis, inflammation and differentiation. Pro-inflammatory cytokines induce the cytosolic release of NF–χβ from its inhibitory Iχβ proteins to allow translocation of NF–χβ into the nucleus and subsequent transcription of proteolytic pathway components (Ladner et al., 2003). In both myocytes and murine models, the cytokine-induced activation of NF–χβ has been shown to inhibit muscle differentiation and function through the suppression of MyoD mRNA and protein (Guttridge et al., 2000). NF–χβ is required to up-regulate the expression of the key E3 ligases (MuRF-1 and MAFbx/atrogin-1) which mediate sarcomeric breakdown and inhibition of protein synthesis (Glass, 2010a,b). MuRF1 is up-regulated in multiple settings of muscle atrophy (Bodine et al., 2001a,b). This E3 ubiquitin ligase is responsible for mediating the ubiquitination of the thick filament of the sarcomere MyHC (Clarke et al., 2007), and other thick filament components (Cohen et al., 2009). MuRF-1 has also been shown to target for breakdown troponin I (Kedar et al., 2004) and actin (Polge et al., 2011), which are components of the thin filament. Furthermore in the latter study it was also shown that MuRF-1 protein levels are up-regulated in muscles of non-cachectic cancer patients. The cytokine TWEAK, in particular, induces MuRF1 upregulation via NF–χβ, resulting in MyHC loss (Mittal et al., 2010). Inhibition of classical NF–χβ is sufficient to decrease significantly tumour induced muscle loss, at least in Yoshida AH-130 ascites hepatoma tumour bearing rats in part, by inhibiting the up-regulation of MuRF1 (Cai et al., 2004; Moore-Carrasco et al., 2007).

MAFbx/Atrogin-1 is a highly specific marker of muscle atrophy as demonstrated in other conditions causing cachexia such as immobilisation, denervation, and glucocorticoid excess (Bodine et al., 2001a,b; Gomes et al., 2001). MAFbx up regulation occurs mainly via p38 activation (Li et al., 2005). MAFbx causes the ubiquitination of eIF3f which is directly involved in skeletal muscle protein synthesis (Lagirand-Cantaloube et al., 2008). Thus MAFbx may provide a key link between the simultaneous regulation of both protein degradation and synthesis in certain models of cachexia (Smith and Tisdale, 1993). Another E3 ligase, Fbxo40 has been implicated in skeletal muscle atrophy (Shi et al., 2011). It has been suggested Fbox40 induces the ubiquitination of IRS1 therefore decreasing protein synthesis. Up regulation of Fbox40 has been seen in denervation but, at present little evidence suggests this may occur in other models of cachexia (Shi et al., 2011).

Administration of anti murine IL-6 receptor antibody to C26 bearing mice reduced weight loss of the gastrocnemius muscle to 84% of that of control mice. The enzymatic activity of cathepsin B + L and mRNA levels of cathepsin L and poly-Ub were significantly depressed in control mice compared with C26 bearing mice, indicating that both the lysosomal cathepsin pathway and the ATP-dependant proteolytic pathway might be involved (Fujita et al., 1996). The direct regulation of E3 ligase expression during IL-6-induced muscle atrophy is controversial (Bodell et al., 2009).

Although less evidence exists to demonstrate that IL-6 can lead directly to lipid mobilisation or altered skeletal muscle protein turnover, there is general acceptance from mouse models (Bonetto et al., 2011) that IL-6 is produced from activated macrophages and may contribute to net muscle loss in cancer cachexia by stimulating the liver (Castell et al., 1989) to induce an acute phase protein response and subsequent reprioritisation of peripheral protein metabolism. The IL-6 receptor signals via the Janus associated kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway (Fig. 3). Downstream activation results in STAT phosphorylation and translocation to the nucleus to regulate genetic transcription (Ihle et al., 1995).

5.1.1.2. TGF-β family activating the ubiquitin proteasome pathway. A more recent factor, which has captured the attention of many investigators, is the TGF-β family member, myostatin. Genetic null animals for myostatin, demonstrate dramatic muscle hypertrophy (Mosher et al., 2007). Myostatin is synthesised and secreted mainly from skeletal muscle cells. Myostatin acts firstly by signalling through the activin type II receptor, which then recruits an Alk family kinase, resulting in the activation of a SMAD2 and SMAD3 transcription factor complex (Sartori et al., 2009; Trendelenburg et al., 2009) (Fig. 3). Secondly, Akt and downstream TORC1 pathways that promote synthesis are down regulated (Trendelenburg et al., 2009; Lokireddy et al., 2012). Myostatin excess causes an increase in skeletal muscle atrophy (Zimmers et al., 2002), whilst inhibition causes a marked increase in muscle mass and myofibre size (Bogdanovich et al., 2002; Whittemore et al., 2003; Lee et al., 2005; Welle et al., 2007). Myostatin antagonism has been shown to prevent cancer cachexia in the LLC mouse model (Benny Klimek et al., 2010). Activin A and B are other members of the TGF-β family that have been found to be up regulated in skeletal muscle after activation of the TNFα/TAK-1 signalling pathway (Trendelenburg et al., 2012). Both Activin A and Activin B are potent growth and differentiation factors with a broad spectrum of biological effects, including modulation of embryogenesis, neuroprotection, apoptosis, and fibrosis. These TGF-β molecules commonly bind to the ActRIIB receptor to initiate a signalling cascade leading to increased expression of MAFbx/Atrogin-1 and MuRF1 and subsequent degradation of myofibrillar proteins (Glass, 2010a,b). Direct evidence linking muscle wasting to survival of the host with cancer cachexia has been lacking until recently (Zhou et al., 2010; Aversa et al., 2012; Busquets et al., 2012; Ju and Chen, 2012). As mentioned previously, Zhou and co-workers found C26 bearing mice that preserving muscle mass is vitally important in the organism's survival: ActRIIB antagonism prolonged survival and prevented muscle wasting and reversed muscle loss. Further work demonstrated that ActRIIB pathway activation induced the ubiquitin ligases critical in muscle wasting and enhanced ubiquitination of muscle proteins, and these effects were completely abolished by ActRIIB antagonism. It was also shown that ActRIIB-SMAD signalling stimulated FOXO3 activity in muscle, which induced transcription of MAFbx/Atrogin-1 and MuRF1. Finally ActRIIB antagonism was shown to dramatically stimulate satellite cell proliferation, which presumably also contributed to the observed rapid reversal of muscle loss in treated animals.

5.1.1.3. Deubiquitinating enzymes. Protein ubiquitination is a reversible process. Much attention has been focused on identification of the enzymes responsible for conjugation of ubiquitin to proteins. However, deconjugation of ubiquitin plays an important role in regulating ubiquitin-dependent pathways (Kim et al., 2003; Wing, 2003). Previous studies have shown increased levels of ubiquitination of muscle proteins in several catabolic conditions including cancer (Wing et al., 1995; Tiao et al., 1996; Lecker et al., 1999; Combaret et al., 2002). In Wistar rats bearing the Yoshida sarcoma, administration of a xanthine derivative close to pentoxifylline that inhibits TNF production, torbafylline (also known as HWA 448) prevented muscle wasting by suppressing elevated proteasome – dependent proteolysis and accumulation of Ub-protein conjugates (Combaret et al., 2002). However, the deubiquinating enzyme USP 19 was shown to be overexpressed in two models of rats bearing the Yoshida sarcoma or hepatoma (Combaret et al., 2005). Whether suppression of deubiquitination contributes to cancer cachexia remains unresolved.

5.1.2. Autophagy/lysosomal pathway

Intracellular lysosomes contain a number of enzymes required for the digestion of macromolecules, including proteases, carbohydrases, lipases and nucleases. Cathepsins L, B, D and H are the major lysosomal proteases and determine primarily the proteolytic capacity of lysosomes (Tisdale, 2009). Cathepsin-L, in particular, is induced early in catabolic states, and levels correlate with increased protein breakdown. Increased cathepsin activity has been demonstrated in various models of muscle wasting, including cancer cachexia, diabetes mellitus, dexamethasone-induced atrophy, disuse, fasting and muscular dystrophy (Helliwell et al., 1998; Wang et al., 1998). The autophagy pathway is necessary to drive substrates to lysosomes, and lysosomal proteolysis is dependent on the activity of cathepsins (Attaix and Bechet, 2007). In a recent study, autophagy was shown to be increased in the muscle of mice implanted with the C26 tumour (Penna et al., 2013). In most mechanistic studies, induction of autophagy has been associated with increased activation of FOXO3A-dependent transcription of autophagy-related genes such as *LC3B* and *BNIP3* (Mammucari et al., 2007) (Fig. 3). An elevation of total lysosomal protease activities has been observed in muscles and liver of DBA/2 mice inoculated with L1210 tumour cells (Greenbaum and Sutherland, 1983; Tessitore et al., 1993a,b) along with increased muscle levels of cathepsin L mRNA in septic rats (Deval et al., 2001). Autophagy has been demonstrated to play a role in cardiac atrophy associated with cancer cachexia (Cosper and Leinwand, 2011,2012). In the C26 tumour mouse model, autophagy (without UPP activation) in cardiac muscle was demonstrated to be responsible for loss of both myofibrillar and sarcomeric proteins. The findings in skeletal muscle in cancer cachexia models are less clear, some have shown a selective targeting of MyHC (Acharyya et al., 2004), whereas other studies do not support a selective sparing of MyHC (Cosper and Leinwand, 2011,2012). Furthermore, studies into variety of other catabolic conditions appear not to show a selective sparing of MyHC (Baehr et al., 2011; Gomes et al., 2012).

5.1.3. Calpains

Several different calcium-dependent enzyme systems exist, including the calpains. The calpains are a family of intracellular, non-lysosomal, calcium-regulated cysteine proteases that mediate cleavage of specific substrates in a large number of regulatory cell processes (Bertipaglia and Carafoli, 2007). In excessive amounts, calpains are capable of degrading cytoskeletal elements, ion channels, other enzymes, cellular adhesion molecules and cell surface receptors. The ubiquitous calpains 1 and 2 (also called μ and m) have been implicated in both the initial degradation of myofibrillar proteins during muscle wasting (Barta et al., 2005) and the necrosis associated with muscular dystrophy (Tidball and Spencer, 2002). Calpains have also been implicated in the degradation of sarcomeric and cytoskeletal proteins in cultured myotubes (Purintrapiban et al., 2003) (Fig. 3). 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 mouse 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). Ubiquitous calpain activity is under

Table 2
Clinical data on myosin composition of skeletal muscle in cancer cachexia.

Authors	Model	Definition of cachexia	Muscle group	Myosin fiber type	Fibre size (FCSA) (μm²)	Fibre percentage (%)
Eley et al. (2008)	Cancer (*n* = 15)	>5% in 6 months	Rectus	All		↓
Weber et al. (2007)	Cancer (*n* = 17)	>20% in 6 months	Quadriceps	1	↓	↑
				2a	↓	↓
				2x	↓	↓
Schmitt et al. (2007)	Cancer (*n* = 8)	>10% in 6 months	Rectus	All		↓
Weber et al., 2009	Cancer UGI (*n* = 19)	>10% in 6 months	Quadriceps	All	↓	
Zampieri et al., 2010	Cancer CRC (*n* = 10)	N/A	Rectus	2		↓

the control of the endogenous inhibitor calpastatin, and downregulation of calpastatin expression has been suggested as one potential mechanism of muscle atrophy. In contrast, overexpression of calpastatin may have potential therapeutic benefits against muscle atrophy. In animal models, transgenic overexpression of calpastatin can reduce both the atrophy caused by unloading and the necrosis associated with muscular dystrophy (Tidball and Spencer, 2000). There is little evidence in the literature to allow assessment of the role of calpains in models of cancer cachexia.

5.2. Protein synthesis pathways

The primary role of suppressed protein synthesis in cancer cachexia has been suggested by studies with mice bearing the MAC-16 adenocarcinoma. Using L-[4-3H]phenylalanine to label proteins in the gastrocnemius muscle to determine rates of protein synthesis, a significant depression (60%) occurred in animals with a weight loss between 15% and 30% (Smith and Tisdale, 1993). In muscle 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). Two major targets for reduced protein synthesis in cachexia include either inhibition of amino acid uptake or the suppression of RNA expression or translation (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., 1984; Baracos et al., 1995). The concept that skeletal muscle protein synthesis in cachexia is reduced simply due to anorexia alone (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 differential time-dependant activation of pathways (White et al., 2011).

The predominant regulator of skeletal muscle hypertrophy is through stimulation of the PI3K/Akt pathway by insulin or IGF-1 (Bodine et al., 2001a,b; Rommel et al., 2001; Glass, 2010a,b) (Fig. 3). Mice in which Akt is transgenically expressed and inducibly activated in skeletal muscle demonstrate dramatic hypertrophy upon the activation signal (Pallafacchina et al., 2002; Lai et al., 2004; Izumiya et al., 2008), helping to prove that Akt is the pathway that is sufficient to mediate hypertrophy downstream of IGF1 upregulation. Activation of Akt leads to an increase in the mTOR/p70S6K pathways and a rise in protein synthesis. As well as inducing protein synthesis, IGF1 can inhibit skeletal muscle atrophy. In the presence of up regulated IGF1 signalling, the atrophy genes MuRF-1 and MAFbx/atrogin-1 are actively inhibited (Bodine et al., 2001a,b).

5.3. Fat-muscle cross talk

Recent work suggests molecular cross-talk between adipose tissue and muscle that occurs through adipokines and myokines. Integrative physiology in obesity and diabetes has long emphasised the importance of chronic inflammation, increased adipocyte lipolysis, and increased levels of circulating free fatty acids in the adipose–muscle cross-talk that contributes to lipotoxicity and insulin resistance in muscle. Intracellular accumulation of diacylglycerol triggers activation of novel protein kinases C with subsequent impairments in insulin signalling (Samuel et al., 2010). A recent study in mice bearing the IL-6 producing, pro-cachectic C26 tumour showed that genetic ablation of adipose triglyceride lipase prevented the increase in lipolysis and the net mobilisation of adipose tissue associated with tumour growth (Das et al., 2011). Unexpectedly, skeletal muscle mass was preserved and activation of proteasomal degradation and apoptotic pathways in muscle was averted. Ablation of hormone-sensitive lipase had similar, but weaker, effects. Physiologically important and previously unrecognised crosstalk between adipose tissue and skeletal muscle may therefore exist in the context of cancer cachexia (Argiles et al., 2005; Johns et al., 2012; Zechner et al., 2012).

6. Skeletal muscle morphology and function in cancer associated myopenia: clinical data

Results from morphological studies in humans to date are shown in Table 2. The limited data available suggest loss of MyHC and reduction in fibre CSA. The rectus muscle of patients with oesophago-gastric cancer cachexia (Eley et al., 2008) has been shown to lose MyHC content as well as undergo a reduction in fibre size. Similarly, the rectus muscle of patients with colorectal cancer has also been shown to lose MyHC content (Zampieri et al., 2010). In pancreatic cancer patients with cachexia MyHC protein levels were decreased by 45% when compared with controls (Schmitt et al., 2007). It has been suggested, based on MRI CSA muscle measurements and ex vivo muscle biopsies, that cancer-related cachexia is associated with a loss of muscle volume, but not of muscle function (Weber et al., 2009). After normalising measured muscle strength for reduced CSA in cachectic patients, although muscle volume was diminished, the principal ability to generate force was maintained. The morphological basis for this finding seemed to be smaller mean fibre diameter associated with an increased total fibre number per area (Weber et al., 2009). In contrast, in a larger series of upper GI cancer patients, muscle mechanical quality measured by knee extensor strength per unit quadriceps CSA was shown to be reduced. Furthermore the degree of impairment of lower limb muscle mass, quality and function and the impact on quality of life varied with weight-loss and sex (Stephens et al., 2012). Few other studies have been conducted to resolve these findings and human

data into fibre size, type, myonuclear, and protein content remain limited.

7. Mediators of cancer associated myopenia: clinical data

Unlike murine models where various highly specific treatments have been tested (e.g. ActRIIB receptor), there are no licensed treatments for cancer cachexia in humans and therefore the data on mediators and mechanisms are entirely of an observation nature. This naturally restricts the conclusions that can be reached. There has, however, been a recent increase in phase II/III clinical trial activity and results from these trials promise to provide much needed insight.

7.1. Systemic mediators of cachexia

As in animal models, cancer cachexia in humans is considered, at least in part, to result from interactions between the host and the tumour.

7.1.1. The acute phase response

An organism responds to the presence of acute infection, tissue injury, trauma or surgery by mounting an acute phase response (APR), this is designed to help limit tissue injury by the increased synthesis of key defence/repair proteins by the liver. However, in certain circumstances when dietary protein intake is limited and the APR is prolonged or severe, an APR can exacerbate muscle wasting by increasing the demands for certain amino acids to support increased hepatic export protein synthesis.

7.2. Pro-inflammatory cytokines

Pro-inflammatory cytokines, in particular IL-6, IL-1, TNF and IFN-γ, are central to systemic inflammation and the induction of an APR. Cytokine interaction between host and tumour cells within the tumour are thought to activate peripheral blood mononuclear cells (PBMC) passing through the tumour vasculature. Increased pro-inflammatory cytokine release by PBMCs has been demonstrated in cancer patients with evidence of an APR (Wigmore et al., 2002; O'Riordain et al., 1999). Moreover, patients with pancreatic cancer and cachexia appear to have higher intra-tumoural IL-6 expression and this is associated with increased PBMC IL-6 production (Martignoni et al., 2005). Tumour associated macrophages (TAM) 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. 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).

Despite the pivotal role of pro-inflammatory cytokines in the aetiology of cancer cachexia in animal models, human trials to date using cytokine antagonists have not generally been successful at ameliorating muscle loss. The use of infliximab, an anti-TNF-α monoclonal anitibody (mAb), in conjunction with docetaxel was shown to be associated with increased fatigue and worsened QoL scores in NSCLC patients compared with docetaxel with placebo, and therefore the trial was stopped (Jatoi et al., 2010). Neither arm of the study demonstrated palliation of weight loss. A further trial of infliximab with gemcitabine in pancreatic cancer patients was unable to demonstrate a significant improvement in LBM or survival (Wiedenmann et al., 2008), whereas a trial of etanercept, a TNF-α inhibitor, in cachectic cancer patients was not associated with weight gain or improved survival (Jatoi et al., 2007). Trials of pentoxyfilline, a cytokine inhibitor, have also shown little clinical benefit in the treatment of cachexia and anorexia in cancer patients (Goldberg et al., 1995). In human trials of an anti-IL6 mAb early clinical studies in patients with non-small-cell lung cancer has shown it to be safe and well tolerated. Treatment with BMS-945429 improved lung symptoms, reversed fatigue, and a trend towards a decrease in the loss of lean body mass was noted (Bayliss et al., 2011). In another study of patients with cholangiocarcinoma, selumetinib (MAPK1 and IL-6 secretion inhibitor) was shown to promote a gain in muscle mass (Prado et al., 2012).

7.3. Other circulating mediators

7.3.1. 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 (Janssen et al., 2002). In sarcopenia, women with low vitamin D have been shown to have approximately twice the risk of reduced muscle mass and strength and a similar relationship was observed with raised parathyroid hormone levels (Visser et al., 2003). Vitamin D deficiency has been reported in over 80% of cancer patients (Stone et al., 2011) and may be particularly prominent in patients receiving chemotherapy (Fakih et al., 2009). A retrospective study of advanced cancer patients reported 70% vitamin D insufficiency and an association with anorexia and fatigue (Dev et al., 2011). In cachectic cancer patients, vitamin D gene receptor polymorphisms have been linked to a more aggressive form of cachexia (Punzi et al., 2012).

7.3.2. Glucocorticoids

In cachectic pancreatic cancer patients, elevated serum levels of cortisol have been shown, along with elevated cortisol:insulin ratios (Fearon et al., 1998). Glucocorticoids are administered frequently to cancer patients undergoing chemotherapy or radiotherapy. This cancer therapy is often associated with exacerbation of muscle wasting. Glucocorticoid atrophy seems specific to type II fibres. Mechanism may involve upregulation of protein degradation pathways, upregulation of myostatin and enhanced glutamine synthetase activity (Carballo-Jane et al., 2004). Glucocorticoids inhibit physiological secretion of growth hormone and appear to reduce IGF-1 activity in target organs.

7.3.3. Insulin

Insulin can stimulate protein synthesis via the PI3K/Akt pathway. Both low insulin production and peripheral insulin resistance has been demonstrated in cancer cachexia (Tisdale, 2009). In cancer patients, treatment with low dose insulin therapy as part of a combination therapy regimen resulted in a rise in carbohydrate intake, and increase in body fat, and a fall in serum-free fatty acids. Despite these findings however, there was no change in lean body mass, maximum exercise capacity or spontaneous physical activity (Lundholm et al., 2007). Higher serum insulin did not change IGF-I levels, which may explain the dissociated effects by insulin on whole body fat and lean tissue.

7.3.4. Ghrelin

A randomised controlled trial has investigated the effects of intravenous ghrelin taken at both low dose and high dose in patients with a variety of advanced metastatic cancer. No difference between treatments was seen for nutritional intake, symptoms, adverse effects or tolerability (Strasser et al., 2008). In another study, 31 patients with metastatic gastrointestinal cancer were randomised in a double-blind manner to ghrelin at 2 different doses subcutaneously daily for 8 weeks. In the high-dose group (versus the low-dose group) appetite scores were significantly better, and

there was a trend to less fat-free mass loss and improved energy balance. There were no differences in food intake, quality of life or physical activity (Lundholm et al., 2010). The studies to date are small phase I and phase II trials, and therefore, results should be treated with caution. A phase III, randomised, placebo-controlled clinical trial assessing anamorelin hydrochloride in patients with non-small-cell lung cancer-associated cachexia is currently recruiting patients (clinicaltrails.gov/NCT01505764).

7.3.5. Androgens

Testosterone inhibits release of TNF, IL-1, IL-6 from macrophages and stimulates production of IL-10 (Li et al., 1993; D'Agostino et al., 1999; 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). In males with pancreatic cancer, systemic inflammation was associated with hypogonadism which in turn was associated with shortened survival (Skipworth et al., 2011). Despite these findings, trials have not been able to show an improvement with exogenous androgens. A trial of nandrolone decanoate in patients with advanced NSCLC demonstrated a trend for decreased weight loss (Chlebowski et al., 1986). In another study comparing fluoxymesterone with megestrol acetate or dexamethasone, fluoxymesterone was inferior in terms of appetite and weight gain (Loprinzi et al., 1999). Recently, SARMs have received much attention as potential muscle-targeted treatments for cancer cachexia. In humans, Phase I and II clinical trials have shown that SARMs increased LBM and enhanced functional status (Zilbermint and Dobs, 2009). In a recent phase IIb randomised, double blind, placebo controlled study, treatment with enobosarm (GTx-024) was shown to significantly increase lean body mass and muscle function in 120 healthy elderly men and women (Dalton et al., 2011) At present, enobosarm is being assessed in a randomised, placebo-controlled, phase III clinical trial in patients with non-small-cell lung cancer receiving first-line chemotherapy treatment (clinicaltrails.gov/NCT01355497).

7.3.6. Myostatin

In a recent study of myostatin expression in muscle biopsies from non weight losing lung and gastric cancer patients, there was evidence of increased expression in the gastric cancer patients (Aversa et al., 2012). This suggests that in human cancer cachexia, myostatin alterations may vary by primary tumour site. There are at least two ongoing phase II trials of anti myostatin strategies (clinicaltrials.gov/NCT01505530), (clinicaltrails.gov/NCT01433263).

8. Mechanisms of cancer associated myopenia: clinical data

8.1. UPP/E3 ubiquitin ligases

In many acute models of cachexia, including cancer, the UPP is thought to be fundamental in the process of muscle atrophy (Bodine et al., 2001a,b; Gomes et al., 2001). However, human investigations have failed to be conclusive. Studies including patients in ICU 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 (Edstrom et al., 2006; Leger et al., 2006a,b; Doucet et al., 2007; de Palma et al., 2008; Fredriksson et al., 2008; Salanova et al., 2008). Likewise, investigations of UPP activity in quadriceps muscle biopsies 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). In a transcriptomic study of UGI cancer patients, candidate genes including FOXO and ubiquitin E3 ligases, were not related to weight loss. Furthermore, promoter analysis identified that weight loss-associated genes had fewer FOXO binding sites than expected by chance (Stephens et al., 2010) (Table 3). 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 a suggestion that the lysosomal pathway was increased (Jagoe et al., 2002) (Table 3). In contrast, in gastric cancer patients with average weight loss 5.2%, increased UPP activity (determined by measurement of RNA and cleavage of specific fluorogenic substrates) was seen compared with controls, with a further effect with increasing tumour stage, weight loss and lower albumin (Bossola et al., 2003) (Table 3). The same group also demonstrated higher levels of ubiquitin mRNA in gastric cancer patients compared with controls, but there was no relationship with 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., 1999) (Table 3). The authors 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,b) (Table 3) and the MAC-16 murine cachexia model (Khal et al., 2005a,b). However, in these studies, UPP activity seemed to increase after a threshold of $\sim$10% weight loss with a peak between 12% and 19% weight loss.

8.2. Autophagy/lysosomal pathway

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) Table 3. 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) Table 3. This perhaps has some appeal because, although one study detected radio-labelled myofibrillar proteins in lysosomes (Gerard and Schneider, 1979), there is other evidence to suggest that lysosomal proteolysis in isolation is not sufficient to degrade myofibrillar proteins (Lowell et al., 1986; Furuno et al., 1990; Cohen et al., 2009).

8.3. Calpains

Calpains are capable of degrading cytoskeletal elements, ion channels, other enzymes, cellular adhesion molecules and cell surface receptors. Furthermore, calpains cleave the enzyme xanthine dehydrogenase to xanthine oxidase, thus indirectly creating the formation of superoxide radicals. Calpains have been implicated in the degradation of sarcomeric and cytoskeletal proteins in cultured myotubes (Purintrapiban et al., 2003). Gastric cancer patients with minimal weight loss (average 1%), had an increase in rectus 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) Table 3.

8.4. Caspases

Caspases are a group of enzymes usually associated with the induction of cellular apoptosis in response to death signals. There is some evidence for a role for apoptosis in muscle wasting experienced by cachectic patients. Muscle biopsies from weight-losing patients with upper GI cancer showed a three-fold increase

Table 3
Findings of different studies in humans on potential mechanisms of skeletal muscle protein degradation.

	Tumour type	% WL	UPP mRNA	Protea-some activity	Calpain activity	Cathepsin B mRNA
Williams et al. (1999)	GI	0–10%	↑			
Bossola et al. (2003)	GI	>5%		↑		
Khal et al. (2005a,b)	GI	>10%	↑			
Stephens et al. (2010)	GI	>5%	=			
Smith et al. (2011)	GI	0–5%	=		↑	
Jagoe et al. (2002)	Lung	<5%	=			↑

in muscle DNA fragmentation compared with control subjects (Busquets et al., 2007). Furthermore, the increase in DNA fragmentation was associated with a four-fold increase in poly (adenosine diphosphate-ribose) polymerase (PARP) cleavage, indicating the presence of apoptosis (Busquets et al., 2007). No human studies to date have focused on targeting caspases or apoptosis in the prevention of cancer cachexia.

8.5. *Myogenesis*

Decrease in skeletal muscle regeneration may also contribute to the loss of skeletal muscle mass in cancer cachexia. In both health and disease, skeletal muscle is able to undergo an extensive repair process aimed at preventing the loss of muscle mass via activation of satellite cells (Zammit et al., 2004; Cossu and Biressi, 2005). The satellite cell starts off as a myogenic precursor during embryonic development, and upon muscle injury starts proliferating with a variety of genes being regulated. Myogenin is subsequently up-regulated leading to terminal differentiation, followed by a permanent exit from the cell cycle. In a recent study looking at patients with gastric cancer these genes were found to be up regulated compared with a control group (Pessina et al., 2010), whether this suggests potential for muscle regeneration during cancer cachexia is unknown.

9. Future prospects

The heterogeneity between morphology, mediators, and mechanisms of animal models and human cancer cachexia is vast. There are some comparisons that can be drawn from the two but it must be realised that these may represent distinct disease processes. The relatively acute wasting seen in young, metabolically active animal models may have little bearing on the chronic disease patho – physiology seen in cancer patients with chronic wasting. Progress in treating the human form of cancer cachexia can only move forwards through carefully designed large randomised controlled clinical trials of specific therapies with validated biomarkers of relevance to underlying mechanisms.

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REVIEW

Muscle protein kinetics in cancer cachexia

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Purpose of review

Skeletal muscle loss appears to be the most significant event in cancer cachexia and is associated with a poor outcome. The balance between mechanisms controlling synthesis and degradation is fundamental when designing new therapies. This review aims to highlight the molecular mechanisms associated with protein kinetics.

Recent findings

The mechanisms that promote muscle synthesis have been explored in detail recently but moreover have been the mechanisms behind degradation. Specific advances in cellular signalling molecules related to autophagy pathways including signal transducer and activators of transcription-3, activin type-2 receptor, TRAF6, and transcriptomic research have been given special attention in this review to highlight their roles in degradation as well as potential targets for therapeutics. Ways to quantify muscle loss are badly needed for outcome measures, and recent research using radiolabelled amino acids has also been discussed in this review.

Summary

Only by having an appreciation of the complex regulation of muscle protein synthesis and degradation, will potential new therapeutics be able to be developed. This review identifies known targets in molecular pathways of current interest, explores methods used to find novel genes which may be involved in muscle kinetics and also highlights ways in which muscle kinetics may be measured to assess the efficacy of such interventions.

Keywords

cachexia, cancer, degradation, muscle, synthesis

INTRODUCTION

Cachexia affects the majority of patients with advanced cancer and is a significant cause of morbidity and mortality [1,2]. A recent international consensus study defined cancer cachexia as 'a multifactorial syndrome characterized 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 characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism' [3▪▪]. Patients may present with varying degrees of weight/tissue loss, and it is important to consider cachexia as a journey rather than an event.

Although there is depletion of both adipose tissue and lean body mass, it is skeletal muscle loss that has the greatest impact on patients and is associated with a poor outcome [3▪▪,4,5▪]. Skeletal muscle is the major reservoir of body proteins, consisting of around 640 separate muscles which account for 40% of total body weight or 50% of fat-free mass. In healthy adults, skeletal muscle mass is maintained by nutritional status and physical activity, reflecting a dynamic balance between protein synthesis and degradation. A predominance of either will result in muscle hypertrophy or atrophy. Even small changes in protein synthesis or degradation will lead to large protein deficits over time due to the continuous process of protein turnover. The myofibril is the base unit of muscle comprising thick (predominantly myosin) and thin (predominantly actin) filaments. Myofibrillar

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KEY POINTS

- Skeletal muscle loss is one of the most significant events in cancer cachexia and is associated with a poor outcome.
- The balance between mechanisms controlling synthesis and degradation is fundamental when designing new therapies.
- Specific advances in cellular signalling molecules related to autophagy pathways including STAT3, ACTRIIB, TRAF6 and transcriptomic research are potential targets for development of therapeutics.
- Development of novel tracer techniques will enable in-vivo quantification of muscle kinetics which will guide the efficacy and development of molecular therapeutics.

proteins account for more than 50% of protein in skeletal muscle and are integral to muscle contraction and function. The preservation or loss of these proteins will dictate the clinical symptoms and there is some evidence that they are selectively targeted in cancer cachexia [6,7].

This review aims to detail some of the recent advances in muscle protein biology and how emerging techniques and therapies seek to manipulate these complex biological pathways to attenuate skeletal muscle loss.

MUSCLE PROTEIN KINETICS IN THE HEALTHY ADULT

Insulin is the most important endocrine factor controlling skeletal muscle protein synthesis (MPS) in the active, well fed, healthy adult. Reduced peripheral insulin sensitivity is described in terms of reduced glucose clearance by peripheral tissues, predominantly skeletal muscle. Glucose uptake and amino acid uptake are both under insulin control and impaired MPS can accompany reduced insulin sensitivity. In patients with reduced peripheral insulin sensitivity, its normalisation may improve skeletal MPS.

QUANTIFICATION OF MUSCLE KINETICS

The understanding of the molecular biology of muscle wasting has increased significantly recently and this has identified a variety of potential drug targets in the complex pathways that control muscle hypertrophy/atrophy. It has been suggested specific elements of the myofibrillar complex may be targeted early in the wasting process (e.g. myosin heavy chain). Clinical trial methodology is, however, limited by the absence of good biomarkers to predict early response and/or determine the net effect of combination therapy. Measurement of skeletal MPS (and perhaps specifically the myofibrillar protein myosin) may allow not only better understanding of the pathophysiology of muscle wasting in cachexia but also provide an early index of the response to muscle-directed therapy. Measurement of protein FSR involves analysis of the increase in AQ2 protein-bound amino acid (product) enrichment with time with respect to that of the tracer amino acid in the intracellular pool (precursor). Human skeletal muscle myosin FSR is low, of the order of 1% day [8]. Precursors of nonessential amino acids AQ3 can label amino acids intrinsically. Deuterium oxide (heavy water) can be used as an intrinsic tracer. Water mixes fully within an hour, it does not stimulate metabolic processes and eliminates slowly at a predictable rate. Single or multiple oral doses are given. Nonessential amino acids incorporate deuterium during de-novo synthesis. In the case of alanine, four hydrogen atoms come into equilibrium with body water and are incorporated into protein. This was first exploited to study protein synthesis in cell cultures and rodent models [9]. Low levels of tracer may best be measured after isolating and hydrolysing the protein and analysing individual amino acids, most probably alanine. GCMS can detect enrichments down to approximately 0.5 mole % excess in individual amino acids [9]. The newer GC-pyrolysis-IRMS technique can detect ppm changes in deuterium in individual molecules. This technique is most suitable for measuring synthesis in major proteins available at high concentration [10]. Previs *et al.* [11] were the first to use $2H_2O$ and GCMS to measure human albumin synthesis. GC-pyrolysis-IRMS was first shown capable of measuring de novo lipogenesis in a heavy water protocol in human patients [12]. Its use to measure tissue protein synthesis in humans is under development [13]. In contrast, modern proteomics platforms, either high resolution or tandem mass spectrometry can be used to measure stable isotope enrichment in several intact peptides simultaneously. As the labelled amino acids may be minor components of each peptide, minimum enrichments of approximately 5 mole % in that amino acid may be necessary for detection. However, modern instrumentation can detect very low peptide concentrations. Initial studies have been conducted in rodents [14,15]. In summary, GC-pyrolysis-IRMS may be necessary to analyse low tracer enrichment human proteins present at high concentration, whereas proteomics platforms are compatible and may be used to measure to measure peptides at low concentration but with high synthetic rate. GCMS can be applied at

intermediate enrichments. A further consideration is that organic mass spectrometry (GCMS or LCMS) allows isotopomer analysis to verify the labelling pattern of precursor and product [9]. A single oral $2H_2O$ bolus can raise body water up to 2000 ppm excess ^2H (0.2 atom % ^2H excess). Greater enrichments may require multiple oral doses to avoid transient feelings of vertigo or nausea in a small proportion of patients. In adults, water half elimination time of some 7–8 days is typical. Measurement of MPS by $2H_2O$ incorporation can either be made using a single bolus with a slow and predictable decline in precursor enrichment [13] or further boli can be taken on a daily basis [9] to produce a 'pseudoplateau' in body water enrichment. An advantage of the former approach is that total body water can be measured by deuterium dilution simultaneously with protein synthesis. In the context of a study to measure skeletal muscle protein fractional synthetic rate, this presents a means of estimating skeletal muscle mass from its proportion to fat-free mass, which has been validated using MRI data [16], and thus estimating absolute MPS.

MUSCLE PROTEIN KINETICS IN CANCER CACHEXIA

In cachexia, there is ongoing debate as to whether a reduction in protein synthesis, an increase in protein degradation or a combination of both is more relevant. Although evidence continues to emerge, it would appear that many of the relevant pathways are influenced by the presence of systemic inflammation [17–19]. Inflammatory-mediated signalling may limit MPS by several mechanisms. Tumour necrosis factor (TNF) α can activate the transcription factor nuclear factor kappa B (NF-κB), which inhibits the synthesis of the muscle-specific transcription factor MyoD, thereby inhibiting differentiation [20], the TGF-β family members induce muscle wasting downstream of SMAD activation [21▪], and interleukin-6 activates signalling by binding to ligand-specific receptors either in soluble or membrane-bound forms to induce the signal transducers and activators of transcription-1 and transcription-3 (STAT1/3), ERK and phosphatidylinositol 3-kinase (PI3K) AKT pathways [22,23] (Fig. 1). The ERK pathway has been previously implicated in cancer cachexia and muscle wasting [24], and the AKT pathway is primarily responsible for anabolism in skeletal muscle [25].

PROTEOLYTIC PATHWAYS

Many research groups have focussed investigation on mechanisms responsible for skeletal muscle protein degradation in cancer cachexia. Although there are four mammalian proteolytic enzyme systems (the proteasome, lysosomal/autophagy pathway, caspases and calpains), it is the proteasome and lysosomal pathways which have received most attention.

The majority of signalling pathways contributing to muscle atrophy in preclinical models are mediated through activation of the ubiquitin-proteasome proteolytic pathway (UPP) [26,27]. The muscle-specific E3 ubiquitin ligases, MuRF-1 and MAFbx/atrogin-1 are upregulated in animal models of atrophy [28,29], and MuRF1 selectively targets the myofibrillar protein myosin heavy chain resulting in muscle wasting [6,7]. However, the role of the E3 ligases in human cachexia is less well defined. Studies including patients following bed rest, limb amputation for vascular disease, limb immobilisation, COPD, amyotrophic lateral sclerosis and ageing demonstrate both increased and decreased expression of MuRF1 and MAFBx [30–33] and evidence for an increase in human cancer cachexia is lacking [34]. There is also data that suggests dissociation between protein dynamics *in vivo* and activation of UPP signalling in human skeletal muscle [35].

Upstream of the E3 ligases, the insulin-regulated transcription factors FOXO1 and FOXO3a have been shown to increase expression of MuRF1 and MAFbx in animal models of cachexia [36,37]. Furthermore, in both myotube and mouse models of muscle atrophy, several autophagy genes are upregulated appear to be under control of FOXO3 [36–39], linking the autophagy and proteasomal systems. This makes the FOXO transcription factors attractive therapeutic targets and indeed, early evidence of FOXO inhibition in preclinical models is promising [40▪].

In cancer patients, there are few studies relating to the role of the autophagy pathway in muscle wasting. Increased cathepsin D and acid phosphatase activity has been demonstrated in patients with varying tumour types and degrees of weight loss [41], and lung cancer patients undergoing resection were shown to have increased levels of cathepsin B [42]. Likewise, expression of the autophagy-related genes GABARAPL1 and BNIP3 has been associated with the presence of systemic inflammation and/or weight loss [34], suggesting a potential role in the development of human cancer cachexia.

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3

Recent work has focused on STAT3 activation in skeletal muscle and has linked it with muscle

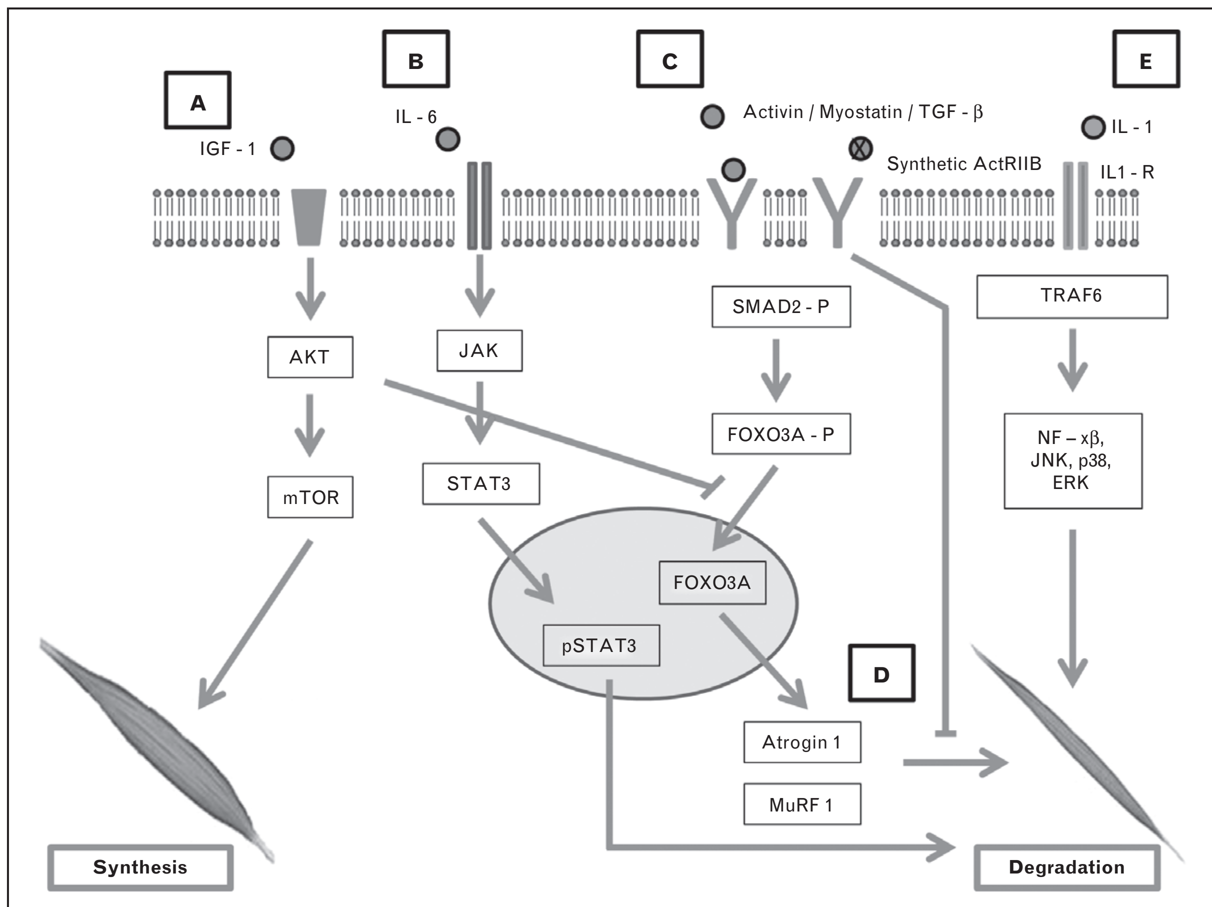

FIGURE 1. Muscle protein kinetics in cancer cachexia. Regulation of muscle synthesis via IGF-1-induced hypertrophy (a). Myokine-activated JAK/STAT pathway involved in the acute phase response and muscle degradation (b). TGF-β-induced SMAD pathway, activated by myostatin and activin (c). Ubiquitin proteasome pathway (d). Activation of TRAF6 (e). ActRIIB, activin type-2 receptor; IL, interleukin; NF-κB, nuclear factor kappa B; STAT, signal transducer and activators of transcription.

wasting and the acute phase response in cancer cachexia [43▪▪]. This contemporary study characterized serum cytokines and the muscle transcriptome in the colon-26 adenocarcinoma model of cancer cachexia. They provided evidence of STAT3 activation, target gene expression and the acute phase response in both liver and skeletal muscle in these mice, providing a molecular link between them. In addition to this, it was also shown that transcription of SOCS3, a classical feedback inhibitor of STAT3 activation was shown to be significantly induced. SOCS3 binds to activated JAKs and inhibits STAT3 activation [44]. However, in contrast to the high SOCS3 mRNA levels in muscle little to 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.

ACTIVIN TYPE-2 RECEPTOR

Myostatin and activin are two members of the TGF-β superfamily that play an important role in growth and are thought to be responsible for the development of cachexia in mice lacking the hormone inhibin [45]. Both molecules bind to activin type-2 receptor (ActRIIB), a receptor in muscle, to initiate a signalling cascade leading to increased expression of atrogin-1 and MuRF1 and subsequent degradation of myofibrillar proteins [25]. Direct evidence linking muscle wasting to survival of the patient with cancer cachexia has been lacking until recently [46,47▪▪,48,49▪]. ~~In~~ one particular study found in murine colon-26 carcinoma-bearing mice that preserving muscle mass is vitally important in the organism's survival. This experimental evidence suggests ActRIIB antagonism prolonged survival and prevented muscle wasting and reversed muscle loss during cancer cachexia. Further work demonstrated that ActRIIB pathway activation induced the AQ4

ubiquitin ligases critical in muscle wasting and enhanced ubiquitination of muscle proteins, and these effects were completely abolished by ActRIIB antagonism. They also showed in the tumour-bearing mice, ActRIIB–SMAD signalling stimulated FOXO3 activity in muscle, which induced transcription of atrogin-1 and MuRF1. Finally, they were able to show that ActRIIB antagonism dramatically stimulated satellite cell proliferation, which presumably contributes to rapid reversal of muscle loss in the treated animals. On the basis of these findings, development of treatments that block these catabolic actions of tumours should be explored as a potential therapeutic avenue.

MYOSTATIN

Myostatin and activin have also both been shown to be expressed 50–100-fold higher in both subcutaneous and visceral adipose tissue in obesity [7]. These results demonstrate that expression of myostatin and activin can be modulated in adipose tissue and skeletal muscle by chronic obesity and suggest that alterations in their expression may contribute to the changes in growth and metabolism of lean and fat tissues occurring during both obesity and cachexia [7]. On the basis of these observations, a therapeutic role for a myostatin antagonist exists and may prove of benefit in patients suffering from cancer cachexia.

TRAF6

TNF receptor-associated factors are proteins involved in activation of various intracellular signalling. TRAF6 is an important E3 ubiquitin ligase, which targets proteins for degradation [50,51]. Several studies have shown TRAF6 to be related to activation of NF-κB, MAPK and PI3K/AKT pathways [52,53]. TRAF6 expression level and autoubiquitination have been shown to be enhanced in skeletal muscle during atrophy in mice [54]. This study also demonstrated that through the activation of JNK1/2, p38 mitogen-activated protein kinase, adenosine monophosphate-activated protein kinase, and NF-κB, skeletal muscle-restricted depletion of TRAF6 rescues myofibril degradation and preserves muscle fibre size and strength upon denervation [54]. Inhibition of TRAF6 also preserves the orderly pattern of intermyofibrillar and subsarcolemmal mitochondria in denervated muscle. This is direct evidence 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.

CANDIDATE GENE IDENTIFICATION

Primary investigations to shed light on the detailed mechanisms that produce cancer cachexia in patients are increasing. With the development of high-throughput technologies such as genome sequencing, microarray gene expression studies and QTL mapping, new hypotheses may be established and subsequently investigated in much more detail than previously possible. This has been based largely on these high-throughput experiments, generating vast quantities of data [55]. In a recent transcriptomics study, the expression of 74 genes correlated positively with weight loss in cancer cachexia patients, and that of nine correlated negatively with it [34]. The most significantly positively correlated gene with weight loss, CAMK2B, encodes calcium/calmodulin-dependent protein kinase II β (CaMKIIβ), a serine/threonine protein kinase that is activated by Ca^{2+}/calmodulin, leading to autophosphorylation and maintenance of CaMKII activity even after the Ca^{2+} signal has diminished [56]. CaMKIIβ is expressed in skeletal muscle. Levels of the protein as well as its phosphorylation status and activity increase after exercise training [57]. In addition, it has recently been demonstrated that Ca(2+)-CaM-eEF2K signalling may be responsible for acute exercise-induced inhibition of MPS, and it is, thus, conceivable that chronic inappropriate activation of this ‘endurance training’-related signalling molecule subdues normal maintenance of skeletal muscle mass. Additional factors that could modulate CaMKII activity include alterations in lipid metabolism [58]. Finally, CaMKII has also been implicated in muscle adaptation through phosphorylation of HDAC5 leading to MyoD/MEF2-driven differentiation of muscle cells [59]. CAMK2B may, thus, have an important role in regulating skeletal muscle function and metabolism. In a recent follow-up study, metabolic and protein turnover-related pathways were found to be suppressed in weight-losing patients with UGIC, whereas removal of the cancer appeared to facilitate a return to a healthy state, independent of changes in the level of physical activity [60▪▪].

CONCLUSION

Circulating mediators in differing concentrations, interplay of synthesis and degradation pathways, and novel gene identification are just some ways in which body composition, particularly involving muscle kinetics are regulated. Although cachectic cancer patients demonstrate systemic inflammation, increased lipolysis and insulin resistance [61], there has been little effort until recently to exploit regulation of muscle mass for therapeutic

gain. Treating clinicians have traditionally focused on curing cancer leaving the cachexia to take care of itself. However, symptom management on its own can improve survival in advanced cancer [62], and multimodal management of cachexia has prompted an increasing number of phase I/II studies using highly specific/potent therapeutics targeted at either upstream mediators or downstream endorgan hypoanabolism/hypercatabolism. Currently, to treat muscle wasting, much of the outcome evidence is based upon observations on loss of muscle mass. However, with the development of novel tracer techniques, quantification of muscle kinetics can be established which will guide the efficacy and development of molecular therapeutics.

The recent work into the regulation of muscle in this review, suggests that understanding of the integrative physiology of this complex system may yet lead to further developments of novel therapeutics, particularly for those patients suffering from clinically relevant muscle loss.

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Conflicts of interest

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AQ5 ~~*None declared*~~.

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■■ of outstanding interest
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Is Tissue Cross-Talk Important in Cancer Cachexia?

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ABSTRACT: Recent work suggests molecular cross-talk between adipose tissue and muscle that occurs through adipokines and myokines. These molecules act in an endocrine fashion to play an intricate role in regulating body composition in both health and disease. Studies in exercise physiology have focused on the molecular cross-talk between adipose tissue and muscle that occurs through adipokines and myokines and on the role these molecules may play in chronic diseases. Similarly, integrative physiology in obesity and diabetes has long emphasised the importance of chronic inflammation, increased adipocyte lipolysis, and increased levels of circulating free fatty acids in the adipose-muscle cross-talk that contributes to lipotoxicity and insulin resistance in muscle. Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life, and duration of survival. Although cachexia in patients with cancer is characterized by systemic inflammation, increased lipolysis, insulin resistance, and reduced physical activity, there has been little effort to manipulate the integrative physiology of adipose tissue and muscle tissue for therapeutic gain.

KEY WORDS: cancer, cachexia, skeletal muscle, adipokines, myokines

ABBREVIATIONS

AA: amino acid; AMPK: adenosine monophosphate–activated protein kinase; ATGL: adipose triglyceride lipase; BMI: Body Mass Index; CRP: C reactive protein; CT: computerized tomography; FA: fatty acid; HSL: hormone sensitive lipase; IL-1: interleukin 1; IL-10: interleukin 10; IL-15: interleukin 15; IL-6: interleukin 6; JAK/STATS: Janus kinases/signal transducers and activators of transcription; LMF: lipid mobilising factor; LPL: lipoprotein lipase; mRNA: messenger RNA; PPAR: peroxisome proliferator–activated receptors; RCT: randomized controlled trial; TNF-β: tumour necrosis factor alpha; VLDL: very low density lipoprotein; ZAG: zinc-α-2-glycoprotein

I. INTRODUCTION

Cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life, and duration of survival. Cancer cachexia has recently been defined 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.[1] However, recent research using animal models of cancer cachexia suggests that mobilization of lipid may not simply be a reflection of a negative energy balance or the effect of tumor-induced lipid mobilizing factors, but may play an active role in regulating loss of skeletal muscle mass.1 In the modern epidemic of obesity there is now a cohort of cancer patients who although obese (not lacking energy reserves) have severe muscle wasting (sarcopenia).[2] These individuals appear to be at increased risk of accelerated demise.

II. NORMAL PHYSIOLOGY

A. Energy Storage and Utilization

After a meal, blood glucose rises and insulin is released. Insulin promotes the storage of glucose as glycogen from the circulation in the liver and muscle. When glycogen stores are saturated, additional glucose is transformed into fatty acids and glycerol, which are synthesized into triglycerides and subsequently stored in adipose tissue. Excess

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amino acids undergo deamination and the carbon chains are metabolized via the citric acid cycle to allow the synthesis of free fatty acids that can be stored in adipose tissue as triglycerides, making adipose tissue the major site of energy storage for all three classes of macronutrients. The third potential store of energy comes from the auto-cannabolism of structural proteins, primarily in muscle. Increased circulating insulin inhibits the release of glucagon and prevents the use of fat as an energy source in preference for glucose. The trigger for glucagon release is a decline in plasma glucose; glucagon stimulates glycogenolysis by binding to G protein coupled glucagon receptors. Glucagon also stimulates the liver to synthesize new glucose (gluconeogenesis). In addition, glucagon regulates the rate of glucose production through lipolysis. When the level of blood glucose exceeds normal limits, insulin is released and allows glucose to be taken up by insulin-sensitive tissues. As the homeostatic mechanisms governing insulin and glucagon play a fundamental role in the central metabolic control mechanism, they are tightly regulated.[3]

B. THE FAST/FEED CYCLE

Storage and metabolism of organic molecules depend upon the body's metabolic state. The major functional metabolic states experienced throughout the day are the feeding (absorptive state) and the fasting (post-absorptive state). During times of feeding and up to 4 hours after, the glucose supply in the blood is plentiful and is the preferred substrate for energy production. Ingested fat and protein (amino acids) are rarely utilized and are more readily stored as glycogen or triglycerides. After four hours, insulin levels decrease and cells switch to fat for energy production, with glucose being reserved primarily for the brain. Blood glucose is maintained via hepatic glycogenolysis. After 24 hours when glycogen stores are depleted, blood glucose is maintained by hepatic gluconeogenesis utilizing substrates including lactic acid, glycerol, and certain amino acids (e.g., alanine). The liver oxidizes fatty acids only to acetyl coenzyme A (acetyl CoA), which is unable to progress through the citric acid cycle for further energy extraction. The liver only partially extracts available energy and converts the remaining molecules into ketone bodies, which may be utilized by peripheral tissues in times of low glucose availability. If fasting continues, the body continues into a state of skeletal muscle and adipose tissue breakdown for fuel utilization. To partially compensate for this, ketone-body production by the liver increases. Ketone bodies are metabolites of fatty acids and may be utilized by the brain. Hence the requirement for glucose decreases with subsequent decline in gluconeogenesis as well as fat and muscle catabolism. When fat reserves are depleted the remaining source of glucose is protein. If this were to continue, the body would progress into a state of auto-cannibalism with eventual breakdown of vital organs. However, in Western society this is rare, given that in a typical day, 3 meals are consumed and little or no absorption is taking place 4 hours after a meal or during the night.[3]

C. INFLUENCE OF EXERCISE ON MUSCLE PROTEIN SYNTHESIS: RELEVANCE TO CANCER CACHEXIA

Physical inactivity results in the accumulation of visceral fat and activation of a network of inflammatory pathways that promote the development of insulin resistance and tumor growth.[4] Exercise has been shown to be beneficial in a number of chronic diseases such as cancer and associated morbidity. Both resistance and endurance exercise exert a number of important effects on skeletal muscle metabolism. Skeletal muscle protein synthesis is highly responsive to its environment. The onset of exercise signals the initiation of transcription of new messenger RNA (mRNA) and encourages the translation of existing mRNA.[5] This short-term change is accompanied by the translation of growth factors and other signaling mediators, which may persist for up to 72 hours, enabling an enhanced anabolic response to exercise.[5] Muscle protein synthesis is increased between two- and five-fold in the post-exercise recovery period of individuals

undergoing resistance exercise for up to 48 hours (in well fed individuals).[6,7] Muscle protein synthesis has been shown to occur in both the myofibrillar and mitochondrial pools in resistance training.[8] Muscle protein breakdown does occur and is at its highest in the post-absorptive state after resistance exercise.[9,10] For endurance exercise, after treadmill walking in the post-absorptive state, an increase in mixed muscle protein synthesis of up to 45% has been documented.[11] Even larger elevations of myofibrillar fractional synthetic rate can be produced by more intense exercise in the fed state. The responsiveness of protein synthesis rates to exercise has been shown to be influenced by physical fitness. Endurance training increases baseline muscle protein synthesis rate and diminishes responsiveness to acute bouts of exercise.[12] Changes observed in both resistance and endurance exercise may not differ greatly.[12] It has been demonstrated there is little difference in muscle protein synthesis rates and responses after acute exercise in muscles of legs working in different modes, i.e., resistance and endurance in the same individual in the untrained state.[8] Muscle protein synthesis is a complex and tightly regulated phenomenon. There would appear to be a transient storage potential immediately after feeding, whereas the post-exercise period stimulates a longer-term adaptive response.[12] Under normal conditions, the body enters a state of anabolism after exercise (via increased protein synthesis) and this is sustained by the ingestion of protein immediately thereafter (via further increased protein synthesis).[8] Obvious phenotypic changes can be observed following specific resistance or endurance training programs. Perhaps the acute anabolic response after a specific type of training influences the type of muscle protein synthesis an individual will undergo. Recent research has shown that cancer cachexia is associated with a loss of muscle volume but not of functionality; furthermore, muscle capillarization is maintained.[13] This suggests that exercise should be encouraged in cachectic patients, as fitness is reduced in cancer cachexia. However, it is unclear how responsive to exercise (in terms of muscle function) cachectic patients are and therefore how successful exercise regimes would be.

III. PATHOPHYSIOLOGY

A. Anabolic Resistance

Previous research has shown that depression of muscle protein synthesis is a major common feature of conditions associated with either slow chronic wasting (cancer, renal failure, chronic obstructive pulmonary disease, and congestive cardiac failure) or acute deterioration (critical illness).[14,15] The phenomenon of anabolic resistance has been proposed, in which there is a reduction in the ability to maintain muscle protein balance by appropriate stimulation of muscle protein synthesis and inhibition of protein breakdown.[5] In the face of a catabolic stimulus and massive protein breakdown, the concurrent rise in free amino acids should stimulate anabolism via synthesis of muscle protein. However, this is not observed in older adults or in immobilized or chronically ill patients.[15] The suggested mechanisms in the elderly include older muscle being less sensitive to the anabolic properties of insulin alongside a decreased capacity for protein synthesis and decreased sensitivity to free amino acids and other anabolic hormones.[15] Anabolic resistance that cannot be overcome by increasing amino acid availability has been shown in immobile or less physically active people.[15] Exercise may help prevent anabolic resistance observed in older adults or in immobilized or chronically ill patients by encouraging muscle protein synthesis and anabolism. Mechanisms proposed include improved amino acid sensitivity and (or) insulin sensitivity of muscle.[16] Recent research has also demonstrated that immobilization induces anabolic resistance in human myofibrillar protein synthesis with low- and high-dose amino acid infusion.[15] Post-absorptive muscle protein synthesis is reduced in the muscle of an immobilized limb. It has been shown that suppressed muscle protein synthesis also extends to the postprandial state, even when the free amino acid concentration is high.

Impaired delivery of nutrients may also play a role in the development of anabolic resistance: two vascular routes within, or closely associated with skeletal muscle exist to provide nutrients. The first route is nutritive; this is closely associated with the muscle architecture and supplies it with amino acids,

hormones, and other substrates. The second functions as a vascular shunt and bypasses the majority of the skeletal muscle tissue. The effect of exercise on the microcirculation has been studied extensively.[17] While there appears to be a strong body of evidence suggesting endurance or chronic training can positively affect structural changes to capillaries, there is conflicting evidence regarding the effects of resistance or exercise training.[17] Recent evidence suggests that the hypertrophic effect of resistance training occurs without an increase in the *in vivo* microcirculation of the skeletal muscles at rest.[18] Blood flow in both resting and exercise conditions is tightly regulated, influencing muscle metabolism and contraction by regulating hormone and substrate delivery as well as product removal. Recent research has demonstrated that nutritive blood flow is an important element of the skeletal muscle response to food and also insulin.[19] Nutritive blood flow is particularly sensitive to low levels of circulating insulin and is recruited at levels lower than that activating glucose uptake.[19] This may be the mechanism by which insulin stimulates muscle protein synthesis and inhibits protein breakdown. Anabolic resistance may be the result of up-regulation of non-nutritive blood flow, effectively becoming the primary source of blood circulation in the critically ill, and resulting in a bypass of skeletal muscle with the nutrients that would normally stimulate protein synthesis.[20] Whether nutritive blood flow is altered in conditions such as cancer cachexia is not known. All these observations need to be taken into context when developing a treatment strategy, as nutrition may not be sufficient to promote net anabolism, especially in cancer cachexia.[21] It is not known whether impaired sensitivity is at all reversible in cancer cachexia.

B. Obesity and Diabetes

The incidence of obesity and diabetes continues to increase worldwide. The obese patient is generating a new challenge in medicine and treatment. The physiology of obese patients differs remarkably from a normal-weighted individual. Recently, patients suffering from advanced cancer have been found to be overweight rather than underweight.[22] This has been shown to confound conventional measurements for risk stratification such as BMI. A recent study of pancreatic-cancer patients has shown sarcopenia when combined with obesity to be an independent adverse prognostic indicator in this patient group and an alternative and more powerful means of risk stratification should be considered.[2] It is, however, unclear how being sarcopenic and overweight/obese causes accelerated demise. The adipokines secreted by excess adipose tissue may act as systemic inflammatory mediators, inducing insulin resistance in skeletal muscle and leading to a further increase in muscle protein loss. Supportive evidence for this hypothesis comes from the observation that in obesity adipocytes swell, increase in size, and eventually die, causing endoplasmic reticulum stress.[23] A chronic inflammatory state ensues secondary to macrophage infiltration, and a cycle of adipose inflammation and insulin resistance results within adipose tissue. The inflammation is set up by a variety of mediators, which act as circulatory factors to regulate insulin sensitivity in distant organs. In obese people, elevated circulating levels of inflammatory cytokines including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), leptin, CRP, and resistin have been detected. These pro-inflammatory cytokines are thought to arise from adipose tissue infiltrated with macrophages in response to low-grade inflammation. Conversely, weight loss is associated with a reduction in macrophage infiltration of adipose tissue,[24] a finding confirmed in a recent study of patients undergoing gastric bypass surgery for weight loss.[25] Thus adipose tissue acts as an endocrine organ, and secreted cytokines can act both locally and at a distance.

Lipotoxicity may also have a role in the metabolic response to obesity. Lipids accumulate in non-adipose tissue (e.g., skeletal muscle) in the presence of high plasma free fatty acids or triglycerides.[26] The excess fat activates a serine/threonine kinase cascade, which has a direct reducing effect on insulin signaling and leads to failure of activation of the GLUT4 transporter to locate to the plasma membrane, thereby initiating insulin resistance.[27]

IV. ADIPOSE SECRETING ADIPOKINES

Adipose tissue, like skeletal muscle, is an active metabolic and endocrine organ. A number of inflammatory cytokines secreted by adipose tissue have been shown with varying effect to influence the development of diseases such as insulin resistance, diabetes, and cancer cachexia by acting on muscle and fat metabolism. Adipokines include IL-6, TNF-α, IL-1, leptin, zinc-α-2-glycoprotein (ZAG) (other name: lipid mobilising factor [LMF]), and resistin.[28]

IL-6 secreted by adipose tissue appears to play a different role from that secreted by skeletal muscle (Fig. 1). In adipose tissue, IL-6 has been shown to be up-regulated in insulin-resistant and obese patients.[4] Visceral adipose tissue secretes more IL-6 than subcutaneous adipose tissue and is the main source of adipose tissue–secreted IL-6. Higher concentrations of IL-6 in the obese population are thought to cause hepatic CRP production, a major risk index for cardiovascular disease.[29] The IL-6 receptor signals via the JAK/STATs (Janus kinases/signal transducers

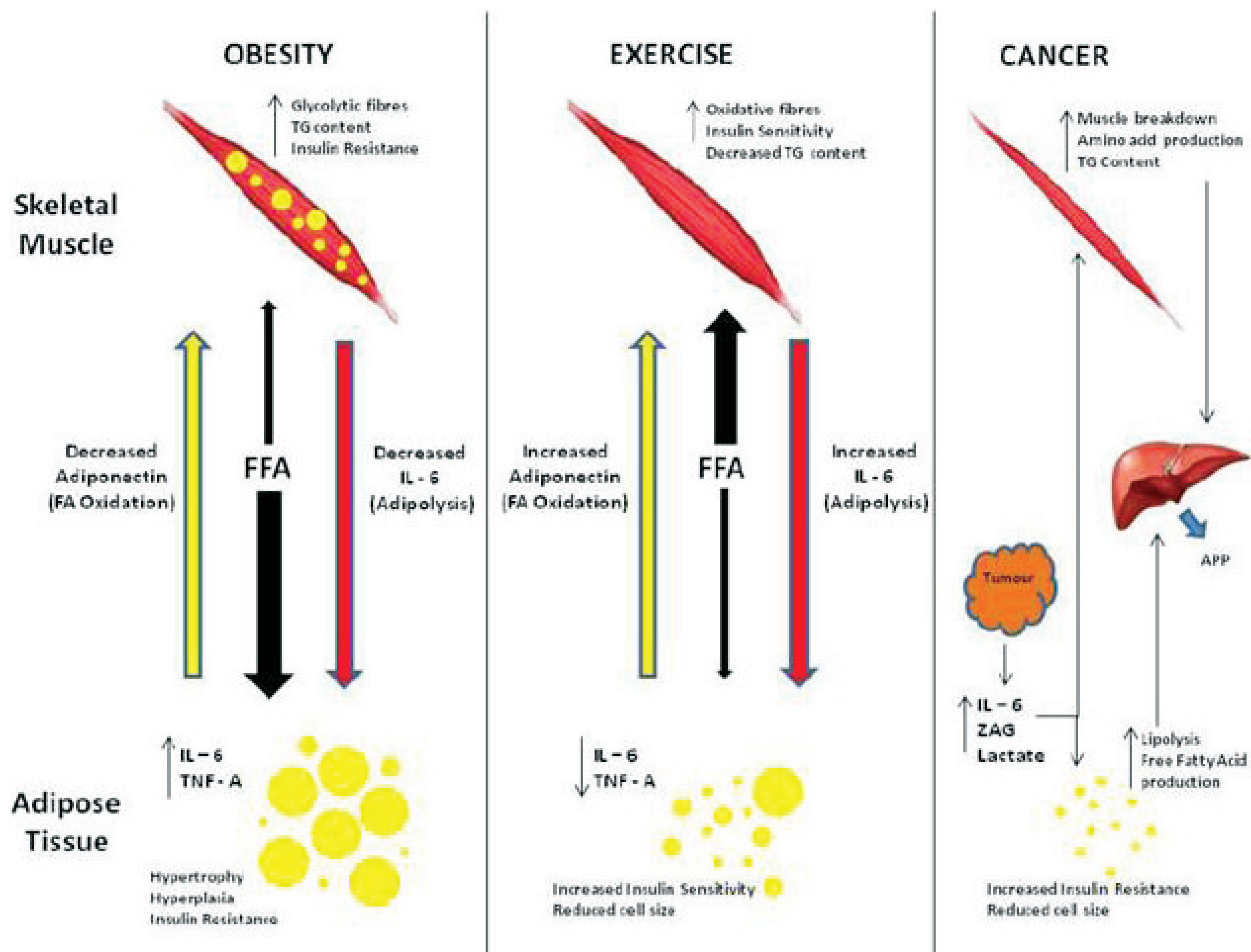

FIGURE 1. Adipose-muscle cross-talk. The main myokines and adipokines involved in the process are shown. Obesity and exercise represent opposite situations concerning expression of these molecules and thus different metabolic balance concerning fatty acid metabolism and insulin sensitivity. In cancer the secretion of the main cytokines leads to degradation of both adipose and muscle mass. FA, fatty acid; FFA, free fatty acid; TG, triglycerides. (Figure adapted from Ref. 73.)

and activators of transcription) signal transduction pathway. Downstream activation results in STAT phosphorylation and translocation to the nucleus to regulate genetic transcription.[30] How IL-6 affects insulin action is not well established; however, it is widely accepted that this adipokine is associated with insulin resistance in the obese patient.[4,23]

TNF-α has been shown to play a role in both animal models and humans in insulin resistance.[31] TNF-α has been shown to initially have a protective role of trying to limit adipose tissue mass by stimulating lipolysis and decreasing LPL expression and activity.[32] TNF-α also has a role in the inhibition of adipose hypertrophy,[33] possibly via paracrine signaling. The latter, however, results in an increase of insulin resistance and prevention of glucose entry into the cell, all mechanisms designed to prevent the adipocyte form swelling. In obesity and insulin resistance, TNF-α may enter a pathologically positive feedback loop, where the production of TNF-α rises in response to increasing lipid deposition in adipocytes and tries to prevent the adipocyte from swelling but thereby increases insulin resistance, which in turn is associated with chronic inflammation and further TNF-α production. The role of TNF-α in obesity and insulin resistance remains poorly explained. TNF-α may well play a role in the inflammatory response to insulin resistance, but secretion from other sources other than adipose tissue may account for this.

Leptin is produced by adipose tissue and acts on the hypothalamus to regulate the amount of energy stored in fat via its influence on appetite. There is little doubt this adipokine has a positive association with BMI.[25,34] A marked reduction of circulating leptin has also been shown as a result of bariatric surgery.[35] Studies of leptin-deficient mice (ob/ob) have demonstrated that this adipokine is fundamental in the control of body weight and lipid metabolism.[36] Experimental administration of leptin has been shown to reduce food intake and promote weight loss.[37] Physiologically, leptin is thought to protect adipocyes from lipotoxicity by inhibiting lipogenesis and increasing fatty acid oxidation.[37] These studies highlight the role of leptin in regulation of body weight. Furthermore, leptin-deficient ob/ob mice have been shown to develop obesity due to lack of appetite suppression. Pathologically, leptin may also act as an adipokine potentiating the chronic inflammation seen in obesity and insulin resistance. Studies have shown that hyperleptinemia is associated with a chronic inflammatory state in the non-obese.[38] Although leptin reduces appetite, levels tend to be exceptionally high in the obese, implying leptin resistance.[39,40]

Adiponectin is produced by the adipocyte and has been shown to decrease insulin resistance. Unlike other adipokines associated with chronic inflammation, adiponectin is inversely related to insulin resistance and BMI. It appears to have protective metabolic and anti-inflammatory properties.[41]

ZAG is involved in the specific mobilization of adipose tissue and increased oxidation of released fatty acids (possibly via induction of uncoupling protein [UCP] expression). ZAG mobilizes fat via the β2-adrenergic receptor. It is produced by both host and certain tumors. Adipose tissue secretes large amounts of ZAG; this is accelerated in cancer cachexia patients and correlates with the reported weight loss.[42]

V. MUSCLE SECRETING MYOKINES

Skeletal muscle, like adipose tissue, is now widely viewed as an endocrine organ, with many studies demonstrating that cytokines produced by muscle fibers exert paracrine and endocrine effects; such cytokines (IL-6, IL-15, and TNF-α) have been labeled myokines[4] and exert metabolic effects on a number of other tissues, including the liver and adipose tissue.

IL-6 secretion via skeletal muscle has been shown to increase during exercise when insulin action is enhanced (Fig. 1). It is proposed that IL-6 acts not only locally, via activation of AMPK in response to muscle contraction, but also has endocrine functions. Intramuscular IL-6 expression is regulated by a network of signaling cascades that are likely to involve cross-talk between the Ca2+/NFAT and glycogen/p38 MAPK pathways. Further studies suggest that IL-6 derived from muscle mediates anti-inflammatory effects, and IL-6 can inhibit TNF production and stimulate synthesis of cytokine antagonists such as IL-1 receptor antagonist (IL-1ra) and IL-10.[4] This

may account for IL-6 being primarily involved not only in local glucose uptake but also in adipose tissue lipolysis. IL-6 is widely known to act as both a pro- and anti-inflammatory cytokine. This is determined mainly by concentration and which tissue it is acting on, whether muscle, adipose tissue, or immune cell.[43]

IL-15 is an important myokine in the regulation of skeletal muscle.[44] IL-15 has a role in the differentiation of myocytes and the ability of muscle fibers to increase the density of contractile proteins.[45] This is confirmed with mouse models, where skeletal myoblast differentiation occurs under certain conditions in response to IL-15.[46] Mechanisms include the transcription factor PPAR-g activating protein synthesis.[47] IL-15 has also been shown to regulate muscle mass in response to resistance exercise training in humans. A recent study proposed that IL-15 is predominantly expressed by type 2 skeletal muscle fibers, and that resistance exercise regulates IL-15 expression in muscle.[48] IL-15 mRNA level was enhanced in skeletal muscles dominated by type 2 fibers and resistance exercise induced increased muscular IL-15 mRNA levels. IL-15 mRNA levels in skeletal muscle were not paralleled by similar changes in muscular IL-15 protein expression, suggesting that muscle IL-15 may exist in a translationally inactive pool.[48] IL-15 has also been proposed to play a role in the prevention of diabetes by modulating glucose uptake in skeletal muscle.[49] A recent study analyzed the effects of IL-15 on glucose metabolism in skeletal muscle. A single dose of IL-15 resulted in a significant increase in glucose uptake in human skeletal muscle, animal models, and in C2C12 cells.[49]

TNF-α has been shown to be released by both adipose tissue and muscle. When acting on muscle, TNF-α has been shown to play a role in muscle protein degradation; it has also been shown to induce muscle cell apoptosis.[50] Release of TNF-α may augment skeletal muscle insulin resistance as well as signaling and potentiating a similar response in adipose tissue.

VI. ADIPOSE-MUSCLE CROSS TALK

IL-6 has a different role to play in exercise compared with insulin resistance/diabetes. Chronically elevated IL-6 levels lead to inappropriate hyperinsulinemia, reduced body mass, and impaired insulin-stimulated glucose uptake in skeletal muscle.[51] Conversely, IL-6 knockout mice develop obesity and insulin resistance, providing evidence against a causative effect of IL-6 in insulin resistance.[52] Clearly IL-6 has numerous roles to play depending on the clinical context; one proposal is that after the peak of IL-6 after exercise, baseline levels return to lower than original and muscle-based receptors are down-regulated.[53] Thus changes in IL-6 levels appear to signify a change between a physiological response and a pathophysiological one.

The myokine IL-15 is an anabolic factor involved in the development of skeletal muscle; it has also been shown to play a part in lipid metabolism.[54] In a study by Nielsen and co-workers,[48] a negative association between IL-15 concentration and trunk fat mass, but not limb fat mass, was observed following exercise. IL-15 secretion from muscle can reduce visceral fat without any change to muscle mass, demonstrating a potent endocrine function.[44] Argiles and co-workers have shown IL-15 acting not just on adipose tissue but on endothelial cells, the gastrointestinal tract, and the liver.[55] IL-15 could have therapeutic roles in the treatment of a number of diseases. In obesity it has been shown to have an anti-adipogenic effect and could be used to prevent lipogenesis. Animal models receiving IL-15 demonstrated a 33% decrease of white adipose tissue mass. This is achieved through a reduction of lipogenesis coupled with a decreased uptake of VLDL triacylglycerol through lipoprotein lipase (LPL).[56] IL-15 facilitates glucose uptake by insulin-sensitive tissues such as skeletal muscle.[55] Argiles and co-workers propose a reciprocal control between adipose and skeletal muscle size. They propose that IL-15 is released from skeletal muscle with the aim of controlling fat deposition and, thus, adipose tissue growth and mass. The anabolic effects of IL-15 have also been shown in animal models.[56] In rats bearing the Yoshida AH-130 ascites hepatoma, IL-15 treatment partly inhibited skeletal muscle wasting by decreasing protein degradation rates to values even lower than those observed in non-tumor-bearing animals. These alterations in protein breakdown rates were associated with an inhibition of the ATP-ubiquitin-dependent

proteolytic pathway. This opens the potential for IL-15 to be used as a therapeutic in wasting diseases such as cancer cachexia.

TNF-α has been shown to play a role in both animal models and humans with insulin resistance.[31] TNF-α may initially have a protective role and limit adipose tissue mass by stimulating lipolysis and decreasing LPL expression and activity.[32] TNF-α also acts to inhibit adipose hypertrophy,[33] possibly via paracrine signaling. This, however, results in an increase in insulin resistance and prevents glucose entry into the cell, all mechanisms designed to prevent the adipocyte from accumulating substrates for energy storage. The role of TNF-α in obesity and insulin resistance remains poorly explained. TNF-α may well play a role in the inflammatory response to insulin resistance, but secretion from other sources other than adipose tissue may be important.

Leptin from adipose tissue is responsible for an increase in fatty acid (FA) oxidation and reduced esterification in resting rodent skeletal muscle.[40] In the absence of leptin, obese human skeletal muscle demonstrates significantly elevated levels of total FA uptake and enhanced rates of FA esterification into triacylglycerol compared with lean subjects.[40] In the presence of leptin, lean muscle demonstrates elevated rates of endogenous and exogenous palmitate oxidation. Leptin also reduces the ratio of esterification to exogenous oxidation, demonstrating the increased partitioning of FA toward oxidation and away from storage. Contrary to these findings in lean muscle, leptin has no effect on FA metabolism in skeletal muscle of the obese. Thus, leptin increases FA oxidation in skeletal muscle of lean, but not obese, humans, demonstrating the development of leptin resistance in obese human skeletal muscle.[40] Leptin has a fundamental role to play in adipose tissue regulation both in health and disease, some of which influences skeletal muscle.

Adiponectin induces fatty acid oxidation in skeletal muscle, and reduced levels are associated with insulin resistance both in mice and humans.[57,58] The proposed mechanism centers around activation of AMP activated protein kinase (AMPK), which is known to regulate cellular malonyl CoA concentrations by inhibiting acetyl CoA carboxylase.[59] Consequently there is a decrease in lipogenesis associated with increased mitochondrial fatty acid beta-oxidation. Adiponectin may also have a role in damping down the inflammatory effects of TNF-α, while TNF-α and IL-6 have a role in decreasing adiponectin levels[60] (Fig. 1).

Adipokines and myokines interact and play a complex role in insulin resistance, obesity, and the regulation of skeletal muscle. It would appear that regulation of any of these adipose-muscle-derived cytokines has a direct effect on the others and on the mass/composition of a variety of tissues in the body. Adipokines have a number of primary and secondary effects throughout the body; however, all seem to be associated directly or indirectly in regulating a chronic inflammatory state, which influences insulin resistance and body composition. Adipokines may also have a role in pre-cachexia, cachexia, and refractory cachexia states. Disruption in the normal physiology of fat metabolism in cancer plays a crucial role in distant organ signaling. On development of insulin resistance, other organs such as the liver, skeletal muscle, and the hypothalamus alter their responsiveness and change how they react to insulin.[31] Therefore, adipose tissue and skeletal muscle can be seen as a producer of cytokines, hormones, and lipids that signal these distant organs to regulate systemic metabolic homeostasis and metabolism. Therapeutic targets may be available to modulate this process. There is little doubt that adipokines and myokines are intricately involved in adipose-muscle tissue cross-talk both in normal physiology and pathophysiology.

VII. PLASMA LEVELS OF CYTOKINES IN CANCER

With the varying roles described in adipose-muscle cross-talk, actual plasma levels of adipokines and myokines will have an important bearing on how they influence body composition in cancer and whether these levels differ in cancer sufferers with or without weight loss. A number of studies have investigated the plasma levels of adipokines and myokines; however, there appears to be great variance in the results obtained[61–65] (Table 1). A recent

study demonstrated adiponectin levels were similarly elevated in cachectic and noncachectic cancer patients compared with noncancer controls. Leptin levels were significantly decreased in cancer cachexia and were found to be associated with lack of appetite and insulin resistance.[61] Contrary to this, adiponectin levels were found to be significantly decreased among a cohort of patients with gastric cancer.[63] Another study demonstrated increased leptin levels in patients with lung cancer.[64] These differences may be a result of inadequate phenotyping in the subjects studied; for example, gender and age differences should be taken into account, as should the type and staging of the tumor. Characterization for body composition (e.g., subtyping for sarcopenic obesity) is another phenotypic variable that is becoming increasingly important, as body composition of adipose and muscle mass will have a significant difference in the circulating levels of adipokines and myokines.

VIII. CANCER CACHEXIA: TARGETS FOR TREATMENT

At diagnosis, one-third of all cancer patients will have lost >5% of their original body weight, and 20% of cancer-related deaths are directly caused by malnutrition and cachexia due to immobility and cardiorespiratory failure.[28] While appetite stimulants or nutritional support can help reverse fat loss, the reversal of muscle wasting is much more challenging and is the core reason why cachexia remains an important unmet medical need. Cancer cachexia results from interactions between the host and the tumor, but the mechanisms behind this interaction are not fully understood. The tumor induces activation of both the acute phase and neuroendocrine stress response. There is a two-way interaction between production of inflammatory cytokines by both host and tumor cells. It is this interplay between a net inadequate nutritional intake and an increasing metabolic demand that is vitally important in cancer cachexia. By understanding the mechanisms involved in normal physiology, the physiology of obesity, and the mechanisms of skeletal muscle loss and fat in disease, a deeper understanding of the mechanisms behind cancer cachexia may be achieved.

A. Nutrition

Skeletal muscle provides a dynamic store of amino acids (AA) that is highly adaptive and responsive to the body and its needs. In the fed state, AA act to stimulate muscle protein synthesis. The converse is true in starvation, when skeletal muscle protein is broken down to maintain the free AA pool.[14] The body's ability to react in this way has been studied extensively.[16,15] Dietary intake of essential AA increases skeletal muscle protein synthesis, whereas non-essential AA does not elicit the same response. Therefore, the ingestion of essential amino acids signals that a meal has been consumed, particularly leucine, which has been demonstrated to act not only as a substrate but also a signal.[66] In the presence of sufficient nutrition, skeletal muscle mass remains constant, with protein balance depending on the time of day and when meals are consumed in line with the fast/feeding cycle. Feeding aids a short increase in the AA pool to compensate for times of fasting rather than modulate longer term phenotypic

TABLE 1. Plasma Levels of Circulating Leptin and Adiponectin in Cancer Patients

Study	Leptin	Adiponectin
Smiechowska et al.[61]	Decreased*	Increased
Kemik et al[62]	Increased	Decreased
Nakajima et al.[63]	—	Decreased
Terzidis et al.[64]	Increased	—
Wolf et al.[65]	Decreased	—

*Leptin levels were significantly decreased in patients with established cachexia.

changes.[14,15] Nutrition has a vital adjuvant role to play in the management of cancer cachexia. Many cancer patients undergoing treatment are malnourished, and a number of interventions such as chemotherapy and radiotherapy further exacerbate this. A multiprofessional and multimodal approach including nutrition and physical activity interventions aimed at preventing or delaying cancer-related malnutrition has been suggested to be of key importance for the efficacy of the oncological therapies.[67] A number of RCTs have also demonstrated that adequate feeding via entral or parenteral methods improves outcome and reduces the side-effect profile of chemoradiotherapy and surgery.[68,69] Cachexia prevention via adequate nutrition remains a primary goal in the treatment of cancer.

B. Importance of Adipose Tissue

The mechanism of fat loss in cancer cachexia centers on the adipocyte. A reduction of the adipocyte size is the primary way fat is lost in cancer cachexia. The lipids, which consist mainly of triglycerides, are broken down by two specific enzymes: adipose triglyceride lipase (ATGL), which is responsible for the formation of diglycerides, and hormone-sensitive lipase (HSL), which activates further hydrolysis to generate the end products fatty acids and glycerol. The activator for lipolysis comes from enhanced production of catecholamines, which are responsible for stimulating and up-regulating these two lipolytic hormones.[70] A recent study examined the therapeutic potential of inhibiting lipolysis through genetic ablation of ATGL or HSL in two murine models of cancer cachexia.[23] These models represent the metabolic end of the cachexia-anorexia spectrum: pro-inflammatory cytokines and ZAG are increased while (during the early and middle phase of tumor growth/cachexia) food intake is normal. They took wild-type C57BL/6 mice and injected Lewis lung carcinoma or B16 melanoma cells to cause tumor growth. In both models, ablation of ATGL prevents the increased lipolysis and net mobilization of adipose tissue associated with tumor growth. Unexpectedly, the authors also demonstrate that skeletal muscle mass is preserved and that activation of proteasomal and apoptotic pathways in muscle is averted. Ablation of HSL had similar but less significant effects.[23]

This study suggests the possibility of until now unrecognized, physiologically important cross-talk between adipose tissue and skeletal muscle in the context of cancer cachexia. The study also raises the possibility of pharmacological inhibition of lipases as a new therapeutic approach to cancer cachexia. This study adds further weight to the argument that lipolysis plays an instrumental role in the pathogenesis of cancer cachexia. The mouse model has limitations; ATGL deficiency in mice and humans is associated with major impairment of cardiac function, which could possibly negate any advantages of a potential therapy based upon ATGL deficiency.[71] Cachexia is a spectrum that has different phases (pre-cachexia, cachexia, and refractory cachexia) and degrees of severity. In this recent study the ablation of lipolysis was present from the onset of tumor growth.[23] Thus the intervention remains untested when, as in clinical practice, the cancer cachexia is often well established. It also remains to be shown whether or not the protection of adipose and muscle loss in lipase-deficient mice is a consequence of defective tissue autonomous lipolysis or due to endocrine signaling from the tumor or adipose tissue. In addition, patients generally receive systemic anti-neoplastic therapy until a late stage in their disease trajectory, and the interaction with such therapy (some of which may induce muscle wasting) is unknown. These issues highlight the importance of understanding the precise mechanism of the findings by Das[23] and colleagues.

C. Importance of Muscle Mass

In the modern epidemic of obesity there is now a cohort of cancer patients who, although obese (not lacking energy reserves), have severe muscle wasting (sarcopenia).[2] These individuals appear to be at increased risk of accelerated demise. In an evaluation of body composition using CT scans in patients with weight-losing pancreatic cancer undergoing palliative therapy, 56% of the patients assessed were sarcopenic, 40% were overweight/obese, and 16% were both. Sarcopenic obesity was shown to be an independent adverse prognostic indicator that should be considered

for stratification of patients entering clinical trials, systemic therapy, or support care programs. This particular toxic combination of excess adipose tissue and minimal skeletal muscle mass is a real therapeutic challenge. The obvious treatment would be to advise exercise, but many advanced, elderly cancer patients are incapable of significant exercise. Thus, the issue of pharmacological intervention is raised. Increased understanding of adipose-muscle cross-talk may be key to identifying suitable targets.

IX. CONCLUSIONS

Although cachectic cancer patients demonstrate systemic inflammation, increased lipolysis and insulin resistance, and reduced physical activity,[28] there has been little effort to exploit adipose/muscle cross-talk for therapeutic gain. Oncologists have traditionally focused on curing cancer, leaving the cachexia to take care of itself. However, symptom management on its own can improve survival in advanced cancer,[72] and multimodal management of cachexia has prompted an increasing number of Phase I/II studies using highly specific/potent therapeutics targeted at either upstream mediators or downstream end-organ hypo-anabolism/hyper-catabolism. The recent work into the cross-talk of organs mentioned in this review, mainly skeletal muscle and adipose tissue, suggests that understanding of the integrative physiology of this complex syndrome may yet further novel therapeutics approaches, particularly for those patients suffering from sarcopenic obesity.

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