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IDENTIFYING THE MOLECULAR MECHANISMS OF EARLY CACHEXIA USING WHOLE TRANSCRIPTOME SEQUENCING IN MUSCLE AND FAT BIOPSIES FROM CANCER PATIENTS

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Thomas E. Adrian

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Declaration of Original Work

I, Amal Hussain Ibrahim Al Haddad, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Identifying the Molecular Mechanisms of Early Cachexia Using Whole Transcriptome Sequencing in Muscle and Fat Biopsies from Cancer Patients*", hereby, solemnly declare that this dissertation is an original research work that has been done and prepared by me under the supervision of Professor Thomas E. Adrian, in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

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Approval of the Doctorate Dissertation

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Abstract

Cancer cachexia is responsible for one third of cancer–related deaths and contributes to the death of many others. More than 80% of cancer patients are cachectic towards the end of life. Despite intensive research, the mechanisms of cancer cachexia are still poorly understood. It is our hypothesis that identification of early changes in gene expression in cachexia will lead to an improved understanding of the mechanism that trigger this important problem in cancer patients.

Thus, to shed light on the mechanisms involved in the major cachexia target tissues, we investigated the entire transcriptome in muscle and fat to identify altered expression of genes in cancer patients with and without cachexia.

Samples of *rectus abdominis* muscle and visceral fat were collected at surgery from patients exhibiting 5-10% weight loss prior to surgery, compared with stable-weight patients. Analysis of all expressed genes was carried out using next generation sequencing (Illumina HiSeq 2500). Also, selected differentially expressed genes were confirmed using real time RT-PCR.

In muscle, 30 genes showed highly significant changes in expression (25 downregulated and 5 upregulated: $P<0.0005$ - $P<0.00001$, FDR 0.2). Analysis of the 25 downregulated genes involved included 7 that are involved with metabolism (5 of which are mitochondrial); 4 with signaling; 4 with ubiquitination; and 3 with intracellular trafficking. There was marked downregulation of multiple genes involved in glycogen metabolism which correlates with the lack of glycogen, muscle weakness, and fatigue; characteristic of cachexia. The 5 upregulated genes include 2 involved with calcium signaling and 2 with cell matrix interactions. Expression of genes previously thought to be important in cachexia, including several inflammatory cytokines, was not significantly different. FBXO32, which encodes atrogin-1, upregulated in an *in vitro* cachexia model, was actually downregulated. No transcripts for the dermicidin gene, which contains the sequence that codes for the backbone peptide of proteolysis-inducing factor, were detected. Expression of myostatin was significantly decreased as was its receptor (ACVR2B), possibly reflecting end organ adaptation to tumor produced myostatin.

In visceral fat, expression of 6 genes were downregulated and 10 upregulated with high statistical significance (P<0.001-0.0002). Several of these encode metabolic enzymes. Of genes in fat previously implicated in cachexia, such as hormone sensitive lipase and adipose tissue triglyceride lipase, were unchanged. In contrast, leptin was significantly downregulated and the zinc-α-2-glycoprotein (lipid mobilizing factor) was significantly upregulated as expected.

These studies confirmed that for a multifactorial condition, genome wide transcriptome analysis is the method of choice to explore the disease complexity. They explain some documented evidence in cachexia pathogenesis, highlight ambiguous data from animal models, and reveal unexpected changes in gene expression that underlie the pathophysiology of the cachectic state in cancer. These results bring reliable, representable, and consistent data from the clinic and back to the bench with more focused insights to be investigated and verified.

Keywords: Cancer cachexia, wasting, skeletal muscle, adipose tissue, RNA sequencing, real-time RT-PCR.

Title and Abstract (in Arabic)

تحديد الآليات الجزيئية للمرحلة الأولية للهزال بدراسة تسلسل كامل التعبيرات الجينية من خزعات عضلية ودهنية من مرضى مصابين بالسرطان

الملخص

الهزال أو الدنف المصاحب للسرطان مسؤول بشكل مباشر عن ثلث وفيات السرطان، وهو أحد المسببات لباقي الوفيات. يعاني أكثر من 80% من مرضى السرطان من الهزال في أيامهم الأخيرة. وبالرغم من الأبحاث المكثفة، فإن آليات الهزال لمرضى السرطان لا زالت مجهولة. لقد تم بناء هذه الدراسة على فرضية أن تحديد متغيرات التعبير الجيني في المراحل الأولية للهزال ستساهم في تطوير الفهم للآليات المحفزة لهذه المشكلة عند مرضى السرطان.

لذلك، فإن الهدف من هذه الأطروحة هو تسليط الضوء على الآليات المسببة للدنف في الأنسجة المستهدفة، عن طريق دراسة كامل التعبيرات الجينية "الترانسكريبتوم" (Transcriptome) في الأنسجة العضلية والدهنية، بهدف تحديد الجينات المتمايزة تعبيريا لمرضى السرطان اللذين يعانون من الهزال، ومقارنتهم بمرضى سرطان لا يعانون منه.

تم جمع عينات من العضلات القطنية (*rectus abdominis*) والدهون الحشوية (visceral fat) أثناء العمليات المجدولة للمرضى الذين خسروا 5-10% من أوزانهم قبل إجراء العملية، وتمت مقارنتها بآخرين من الذين بقيت أوزانهم ثابتة. تم تحليل مستوى التعبير الجيني بدراسة تسلسل كافة الجينات بواسطة تقنيات عالية الإنتاجية (Next generation sequencing) باستخدام جهاز (Illumina HiSeq 2500). كما وتم تأكيد النتائج على مجموعة مختارة من الجينات باستخدام تفاعل البوليميراز المتسلسل اللحظي (Real-time RT-PCR).

أظهرت نتائج الدراسة في الأنسجة العضلية تغيرات هامة للغاية في التعبيرات ل 30 جين (25 منها مثبط، بينما 5 منها مفعل: القيمة الاحتمالية (FDR 0.2, P<0.0005-0.00001). تحليل ال 25 جين المثبط أظهر أن: 7 منها يساهم في عمليات الأيض (metabolism) (5 من هذه السبعة عمليات أيض في الميتوكوندريا)، 4 في تأشير الخلية (signaling)، 4 في عمليات التحليل البروتيني (ubiquitination)، و 3 في حركة سير البروتينات داخل الخلايا (intracellular trafficking). كما أظهرت الدراسة تثبيطا واضحا للعديد من الجينات المشاركة بأيض الجلايكوجين، والتي بدورها تفسر سبب نقص الجلايكوجين بالعضلات وما يصاحبه من ضعف وإنهاك، وهي أعراض أساسية مصاحبة للهزال. في المقابل، فإن تحليل ال 5 جينات المفعلة أظهر أن: 2 منها يساهم في تأشير الكالسيوم، و 2 بتفاعلات نسيج الخلية الخارجي.

من ناحية أخرى، فإن بعض التعبيرات الجينية للسيتوكينات المسببة للالتهاب – والتي كان يُعتقد حسب دراسات سابقة أن لها تأثيرا في الهزال – لم تظهر أي تغيير يُذكر. كما أن الجين الملاحظ تفعيله بالعديد من الدراسات المختبرية (*in vitro*) وهو (FBXO32; atrogin-1) وجد مثبطا. كما لم يتم الكشف عن أي تعبير جيني لجين ال (dermicidin). كما أن التعبير الجيني لجين ال (myostatin) بالإضافة للمستقبل الذي يعمل عليه (ACVR2B)، وجدا مثبطين على غير المتوقع، مما قد يعكس احتمالية وجود تكيف من الأنسجة العضلية لتأثير ال (myostatin) المنتج من الورم السرطاني.

أما بخصوص النسيج الدهني الحشوي ، فقد أظهرت نتائج الدراسة تغيرات هامة للغاية في التعبيرات ل 16 جين (6 منها مثبط، بينما 10 منها مفعل: القيمة الاحتمالية (P<0.001-0.0002). تحليل هذه الجينات أظهر مشاركتها الإنزيمية في العمليات الأيضية. مقارنة نتائج هذه الدراسة بدراسات الهزال السابقة أظهر عدم ظهور أي تغيير على إنزيمات الليباسيس (hormone sensitive lipase and adipose triglyceride lipase). بالمقابل فإن هرمون الليبتين (leptin) كان مثبطا، بينما بروتين (zinc-α-2-glycoprotein) كان مفعلا بشكل واضح، كما هو متوقع.

أثبتت نتائج هذه الدراسات أن الهزال متعدد الأسباب. كما أظهرت أن الطريقة المثلى لدراسة واستكشاف تعقيدات الأمراض هي عن طريق دراسة تسلسل كامل التعبيرات الجينية. هذه الدراسة شرحت بعض الدلائل الموثقة بدراسات سابقة، وألقت الضوء على بعض الغموض في نتائج الدراسات على حيوانات التجارب. كما وأظهرت نتائج غير متوقعة لتطور مرض الهزال لمرضى السرطان. هذه النتائج هي بمثابة نموذج يمكن الاعتماد عليه نظرا لثبات بياناته وتناسقها، والتي تعكس الصورة السريرية لهؤلاء المرضى بشكل أدق، ليتم بحثها و التحقق منها بشكل أفضل مخبريا.

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Dedication

To my beloved parents and dear patients

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List of Abbreviations

AChRs	Acetylcholine Receptors
ACVR2B	Activin Type 2 B Receptor
ADP	Adenosine Diphosphate
AGPC	Acid Guanidiniumthiocyanate-Phenol-Chloroform
AGRN	Agrin
AMP	Adenosine Monophosphate
AMPK	AMP-activated Protein Kinase
APP	Acute Phase Proteins
APR	Acute Phase Response
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
AZGP1	Zinc-α Glycoprotein 1
BAT	Brown Adipose Tissue
BMI	Body Mass Index
CAMs	Cell Adhesion Molecules
CO_2	Carbon Dioxide
COL4A1	Collagen Type 4 Alpha 1
COL4A2	Collagen Type 4 Alpha 2
CRP	C-Reactive Protein
CVD	Coronary Vascular Disease
DAG	Diacylglycerol
DCD	Dermicidin
DGC	Dystrophin-Glycoprotein Complex
ECM	Extracellular Matrix
EPO	Erythropoietin
FC	Fat Weight-Stable Controls
FS	Fat Cachectic Cases
GABA	Gamma-Aminobutyric Acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDF8	Growth and Differentiation Factor 8
Gender M/F	Gender: Male or Female
GH	Growth Hormone
GLUT4	Glucose Transport Type 4
GOI	Gene of Interest
GTP	Guanosine-5'-Triphosphate
IFNG	Interferon-Gamma
IFN-γ	Interferon Gamma
IL-1ß	Interleukin-1ß
IL-6	Interlukin 6
IP_3	Inositol 1,4,5-Trisphosphate
ITGB6	Integrin, Beta 6
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes

LDL	Low Density Lipoproteins
LIPE	Hormone Sensitive Lipase
LTP	Long-Term Potentiation
MAPK	Mitogen-Activated Protein Kinases
MC	Muscle Weight-Stable Control
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
mRNA	Messenger Ribonucleic Acid
MS	Muscle Cachectic Cases
MSTN	Myostatin
NCAM	Neural Cell Adhesion Molecule
NFAT	Nuclear Factor of Activated T Cell
NMJ	Neuromuscular Junction
NMU	Forneuromedin-U
PAL	Physical Activity Level
PHKA1	Phosphorylase Kinase, Alpha 1 (muscle)
PHKG1	Phosphorylase Kinase, Gamma 1 (muscle)
PHKB	Phosphorylase kinase, Beta
PIF	Proteolysis-Inducing Factor
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PITP	Phosphatidylinositol Transfer Protein
PKC	Protein Kinase C
PLC	Phospholipase C
PNPLA2	Adipose Triglyceride Lipase
POMC	Pro-Opiomelanocortin
PP1	Protein Phosphatase 1
PTHLH	Parathyroid Hormone-Related Protein
PTM	Remove
PXN	Paxillin
Real-time RT PCR	Real-Time Polymerase Chain Reaction
REE	Resting Energy Expenditure
RNA-Seq	RNA Sequencing
SLE	Systemic Lupus Erythematosus
STAT5A	signal transducer and activator of transcription 5A
TCF7L2	Transcription Factor 7-Like 2
TEE	Total Energy Expenditure
TNF-α	Tumor Necrosis Factor α
TPPII	Tripeptidyl Peptidase II
TTN	Titin
UCP	Uncoupling Protein
WBC	White Blood Cells

Chapter 1: Introduction

1.1 Overview

1.1.1 History, Etymology, and Definition

Cachexia is known for centuries. The earliest link of cachexia to chronicity of ailment dates back 2400 years to the classical Greece and the school of medicine of Hippocrates [1]. It was described by Hippocrates as:

> "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].
>
> Hippocrates, 460-377 BC

The first time cachexia was used to describe involuntary weight loss in the context of chronic illness is unknown. However, "cardiac" cachexia was first documented by Charles Mauriac, a French physician, back in 1860 when it was described as a:

> “commonly observed secondary phenomenon in patients affected with diseases of the heart... a peculiar state of cachexia which is... conventionally designated cardiac cachexia” [2].

Cachexia was also acknowledged by Herta Müller, a 2009 Nobel Prize winner for literature, who wrote in her novel "Atemschaukel", which was translated to English "The Hunger Angel"[3] as:

> "…once the flesh has disappeared from the body, carrying the bones becomes a burden; it draws you down into the earth”
>
> Müller, 2009

Cachexia is a term originating from the Greek " kakós" and "hexis"; meaning "bad condition or appearance" [2]. Cachexia or the "Wasting Syndrome" is evident in many diseases and conditions such as: cancer, chronic heart failure, chronic kidney disease, chronic obstructive pulmonary disease, cystic fibrosis, rheumatoid arthritis,

multiple sclerosis, Alzheimer's disease, infectious diseases such as acquired immune deficiency syndrome, and tuberculosis, familial amyloid polyneuropathy, gadolinium poisoning, mercury poisoning, hormonal deficiency, and many other chronic illnesses [4].

Definition of cancer cachexia has changed several times in the last few years despite the slow-paced understanding of its etiopathogenesis. Up till 2008, cachexia had no operational definition that can adequately and reasonably validate it in terms of its existence and pathological processes. This had hindered, for years, properly designed and conducted clinical and basic sciences research. Thus, many efforts were taken to reach a consensus definition for the syndrome [5-7]. However, many cachexia definitions share two main themes; weight loss and inflammation [8] with many other associated symptoms: anorexia, fatigue, insulin resistance, and increased muscle protein breakdown. Cachexia is different from starvation, age-related loss of muscle mass, primary depression,malabsorption, hyperthyroidism, and all other metabolic diseases and conditions. The latest definition of cancer cachexia states that it is 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" [7].

None of the definitions of cachexia suggested so far are final. However, the most recent systemic review indicated that in clinical practice, muscle wasting is assumed in relation to weight loss in cancer patients, provided the patients have no fluid retention or a large tumor mass [9]. Keeping in mind that this thesis wishes to identify mechanisms of early cachexia, we operationally defined early cancer cachexia to guide our patients' selection as: cancer patients with documented or self-reported weight loss of 5-10% of total body weight in the past 6 months.

1.1.2 Epidemiology

Cachexia is one of the most serious and highly prevalent, however, underestimated and under-diagnosed syndrome. Cachexia is a major unmet health challenge. Despite of the scarce resources about the extent of cachexia, it is believed that the overall prevalence of cachexia is growing worldwide. It is estimated to constitute around 1% of the total population in the developed world [4] and a possibly comparable number in the rest of the world. Cachexia prevalence is generally high and variable amongst different pathological conditions. For example, it ranges from 5% to 15% in congestive heart failure and chronic obstructive pulmonary disease [4], while it reaches 60% to 80% in advanced cancer [4, 10]. Data about prevalence of cachexia in the cancer patients varies considerably depending on the cancer type and stage, and also differed depending on the criteria used for its diagnosis [11].

The incidence of cachexia among cancer patients is very high, but it is dependent on tumor type and the stage of the disease. For example, the incidence in patients with gastric or pancreatic cancer is more than 80%, while it is around 50% in lung, prostate or colon cancer patients, and approximately 40% of patients with breast cancer or some leukemias [12, 13]. However, tumor type was not found to have a direct correlation to extent of cachexia. For example, some lymphomas can weigh several kilograms and cause no cachexia, while pancreatic tumors as small as <2 $cm^3$and weighing one or two grams may cause profound cachexia.

1.1.3 Classification of Cancer Cachexia

Cancer cachexia is classified to three clinical stages; precachexia, cachexia, and refractory cachexia. Each of these stages has distinguishing clinical features agreed on via consensus panel of experts in the field [7]. Stages are distinguished primarily

on body mass index (BMI) as an indication of body energy stores in addition to the general overall condition. In precachexia, patients demonstrate substantial involuntary weight loss (i.e. ≤5%) in addition to early clinical and metabolic signs (e.g. anorexia). In cachexia stage, weight loss exceeds 5% of stable body weight over the past 6 months, or a body mass index (BMI) less than 20 kg/m² and ongoing weight loss of more than 2%. On the other hand, refractory cachexia is a stage reached at the very advanced cancer when patients are clinically "terminal" (life expectancy of less than 3 months) and it is distinguished by the presence of rapidly progressive cancer unresponsive to antineoplastic therapy, active catabolism, and/or the presence of factors that render active management of weight-loss no longer possible or appropriate [7]. In refractory cachexia, patients also demonstrate low performance status. While there are three identifiable stages of cachexia, patients will not necessarily exhibit the all three phases.

The risk of developing cachexia, the stage of cachexia, and the rate of progression of cachexia varies depending on the cancer type and stage, the presence of systemic inflammation, food intake, and response to anticancer therapy [7]. Cancer cachexia is further complicated by anticancer treatment (e.g. chemotherapy and radiotherapy) which themselves induce anorexia, and malabsorption that exacerbate the effects of the cancer itself. Also, obstruction of the gastrointestinal tract by the tumor will also cause anorexia, nausea and vomiting and reduction in digestion and absorption. All these can even further complicate cachexia.

1.1.4 Significance of Weight Loss in Cancer Patients

Cachexia is known since ancient Greek times as a label for 'signummaliominis' (indicating end stage and poor quality of life) in various, mostly fatal, diseases. Weight loss (the most prominent feature of cachexia) is a positive risk factor for

death for cancer patients. Its presence is always associated with high-mortality and diminishing quality of life [14]. Mortality rates of patients with cachexia are highest in cancer patients. More than 30% of cancer patients die due to cachexia and it reaches around 80% in some cancer cases, and more than 50% of patients with cancer die with cachexia being present [4, 15]. In cancer patients, death normally happens when weight loss exceeds 30% of total body weight [16]. While in starvation, weight loss exceeding 40% of body weight usually leads to death [17]. Considering cancer as a disease of old age, weight loss is a well-documented factor for a significant increasing mortality among elderly patients whether they are discharged home [18] or residing at nursing homes [19].

On the other hand, weight loss as a primary measure of cachexia is important due to unavailability of data on body composition. Attainment and accurate measurement of baseline data on body composition is clinically hard to perform and isn't yet considered as an acceptable clinical standard. Also, changes in body weight can be accurately and easily assessed and followed up.

On the other hand, however, weight loss is still debated as a mono measure of diagnosing the cachectic state. Weight loss alone cannot fully explain the associated physical symptoms that accompany cachexia. Nevertheless, the preponderance of evidence is in favor for the importance of weight loss as the main clinical manifestation of cachexia and that it is highly predictive of morbidity and mortality in cachectic patients.

1.1.5 Diagnosing Cancer Cachexia

Cancer cachexia is rarely screened and always under-diagnosed due to its complexity in nature, and the lack of generally acceptable definition or diagnostic guidelines. However, clinically, multiple definitions of cachexia been utilized to

examine the presence or absence of cachectic state in cancer patients. Fox et.al. (2009) conducted a study to estimate the cachectic state amongst cancer patients based on four different cachexia definitions; ICD-9 diagnostic code of 799.4 (cachexia), ICD-9 diagnosis of cachexia, anorexia, abnormal weight loss, or feeding difficulties, prescription for megestrol acetate, oxandrolone, somatropin, or dronabinol, and ≥5% weight loss in patients with different malignancies [11]. The eye opening findings of the study was the huge discrepancy in cachexia prevalence in cancer patients under each of these categories depending upon the employed definition. For example, in breast cancer patients, prevalence of cachexia was 8.8%, 13.9%, 20.5%, and 31.3% for the four categories respectively. That is around 3.6 fold increase between the first and the forth definition [11]. Thus, unless we operationally define cachexia based on more educated insights about its etiopathogenicity, developing a specific/sensitive/accurate diagnostic measure/s is an unattainable goal.

The two gold standard measures for diagnosing cachexia should be computed tomography and the magnetic resonance imaging. The two measures are expensive and not used routinely. However, since cachexia is a time-dependent process, it is highly unlikely that these methods could be routinely applied to the clinical diagnosis of cachexia, because of the absence of a baseline comparison.

1.2 Management of Cancer Cachexia

Patients with cancer cachexia have limited treatment options. Current available options basically treat symptoms of cachexia, such as anorexia, and not cachexia itself.

1.2.1 Nutritional Treatment

Many attempts were made to reverse cachexia via nutritional management (i.e. enteral, parenteral, and nutritional supplements). However, none of these achieve improvement in lean body mass [20]. A meta-analysis evaluating the effect of enteral nutritional supplementation failed to demonstrate any survival benefit in cancer patients but was able to decrease some post operatively risks and to reduces the overall hospital stay [21]. Furthermore, parenteral nutrition was found to be associated with net negative consequences in cancer patients [22] and does not alone affect the overall survival or quality of life [23].

Supplements tested in cachexia include minerals, vitamins, omega-3fats, protein-enriched nutrition, and antioxidants [24]. Omega-3fats, have been extensively investigated in cachexia and found to have a minimal positive effect on weight, appetite and quality of life in advanced cancer patients[25]. Taken together, the complex mix of different mediators involved in the pathophysiology of cancer cachexia renders nutritional intervention a strategy unlikely to succeed completely, although of course overall nutrition needs to be adequate [26].

1.2.2 Non-Pharmacological Treatments

Non-pharmacological treatments include different measures such as: counseling/education, psychotherapeutic interventions, and physical training (or therapeutic exercise). Psychotherapeutic interventions such as the creation of a pleasant environment for meals, encouraging patients to eat with adequate attention to the food preferences of the patient show some promise [27]. The pathophysiologic changes in cachexia include: insulin resistance, a decrease in the rate of protein synthesis, and a systemic inflammatory response. Physical exercise

exerts beneficial documented evidence in increasing insulin sensitivity, protein synthesis rate, and suppression of the inflammatory response [28]. However, most research has not found a conclusive evidence for its effectiveness [29].

1.2.3 Drug Therapy

Many drugs have undergone clinical trials that attempt to reverse cachexia. Thalidomide was used in clinical trial with advanced pancreatic cancer patients and found to be well tolerated and effective at attenuating loss of weight, arm muscle mass and physical function [30]. Nevertheless, no study has provided information on long-term weight gain in a larger trial, or in end stage disease, or in advanced cachexia. On the other hand, cannabis extract of tetrahydrocannabinol did not result in any differences in appetite or quality of life in patients with cancer cachexia [31]. While cannabinoids may increase appetite in selected patients, there is not enough evidence to support their use. In a systematic review, megestrol; an appetite stimulant, was found to improve appetite and weight gain in cancer cachexia [32], but it increased mortality without a significant increase of body-weight [33].

Short-term treatment with steroids improved appetite and activity in terminally ill cancer patients [34] while in longer treatment duration did not result in weight gain [35-37]. On the other hand, the long-term use of non-steroidal anti-inflammatory drugs showed reduction in the resting energy expenditure and preservation of total body fat, while lean body mass was not influenced [38]. Finally, prokinetics did not improve the nutritional status of patients with cachexia [39].

1.2.4 Multimodal Therapy

Considering the multi-factorial nature of cancer cachexia pathogenesis, treating the underlying neoplasm that trigger its initiation is the treatment gold standard. The

best way to treat cancer cachexia is to cure the cancer. Anticancer treatment will not, per se, alleviate cachexia through targeting the metabolic changes and the other documented etiologies. Metabolic changes such as reduced energy intake, in turn, can be supported by targeting more than one of the contributing factors despite the absence of properly tested combination therapy in cancer cachexia. For example, combination of megestrol with tetrahydrocannabinol [40] and eicosapentaenoic acid [41] did not provide any benefits compared with megestrol alone but was more effective when combined with ibuprofen [42]. Another large study comparing medroxyprogesterone, megestrol acetate, eicosapentaenoic acid, L-carnitine and thalidomide found that the combination of all drugs was superior to any of the other treatment arms with single drug treatment leading to increased lean body mass, decreased resting energy expenditure and improved appetite [43]. Therefore, multimodal therapy for cancer cachexia should be presented as opposed to monotherapy. Also, early palliative care should be initiated to manage the individual symptoms associated with refractory cachexia and that research in this domain should be promoted.

1.3 Prevention of Cachexia

Many intervention been tested for likelihood to prevent cachexia. Relaxation training with deep abdominal breathing, autosuggestion, controlled tension and relaxation of body parts and voluntary image control are some of these tested interventions. Relaxation, for example, was found to have positive effects on body weight and performance status in a group of cancer patients [44]. However, this study did not separate the cachectic from the non-cachectic patients and its results have not been reproduced.

1.4 Etiopathogenesis of Cancer Cachexia

Cancer cachexia is a multifactorial, so far, irreversible syndrome. Symptoms of cachexia involve loss of muscle, with or without fat, frequently associated with anorexia, inflammation and insulin resistance. Cachexia has its unique pathology that sets it unique compared to starvation. Cachexia has a preferential muscle loss for an equivalent amount of weight loss compared to starvation [45]. In cachexia, the visceral protein compartments are preserved [46]. Muscle loss occurs predominantly in skeletal muscles rather than visceral muscle [47]. Even more interestingly, the volume of the liver and other viscera may increase in cancer cachexia as opposed to the decrease observed in starvation [45]. However, the mechanisms of that increase are unknown. As in sepsis, patients with advanced cancer have an elevated rate of whole-body tracer flux and depressed non-export (structural) hepatic protein synthesis rate [46]. This brings cancer cachexia in proximity to sepsis. Yet, cancer cachexia develops slowly and is not associated with the tremendous increase in basal metabolism due to the surge of different inflammatory cytokines in sepsis [48].

On the other hand, patients with anorexia have the majority of weight lost from fat compared to lung cancer patients, who lose weight from both, total body fat and skeletal muscle protein mass [49]. In summary, the changes in body composition seen in cachexia resemble those found in infection and injury rather than those in starvation. During prolonged starvation, ketone bodies produced by fat metabolism in the liver replace glucose as an energy source for the brain, thus preventing loss of muscle through glucogenesis from amino acids. However in cachexia, this does not occur, probably because the energy demands on the host are sufficiently high to

prevent the buildup of acetyl CoA in the liver and its subsequent conversion to acetoacetate and -hydroxybutyrate [50].

1.4.1 Energy Balance and Energy Expenditure

Body mass is controlled by the balance between the amount of energy intake and energy expenditure. Therefore, a major contributor to the cachexia can be attributed to the increased energy expenditure. Since the majority of energy intake is spent on the resting energy expenditure (REE), determining the level of REE in cancer patient can be the starting point to uncover the etiology of cancer cachexia. The rate of REE in cancer patients is strongly determined by the type of tumor. For example, patients with lung [38] and pancreatic [51] cancer have an elevated REE, while gastric and colon cancer patients have no increase in REE [38]. This can, at least in part, explain the higher mortality rates of lung and pancreatic cancer patients. In pancreatic cancer patients, for example, the increase in REE is proportional with the increase in the acute phase response (APR) [51]. APR proteins have long been known to be a measure of underlying tissue injury, infection, or inflammation. In APR, the liver redirects protein synthesis from albumin to acute phase proteins (APP), such as C-reactive protein (CRP), fibrinogen, serum amyloid A, α-2-macroglobulin, and andα1-antitrypsin. Elevated levels of APP, in turn, are correlated with shorter survival in pancreatic cancer patients [51]. The link between the development of an APR and the rate of loss of body mass is also established in lung and gastrointestinal cancers [52]. Furthermore, serum levels of interleukin6 (IL-6) was found to correlate with level of CRP in patients with non-small cell lung [53]. Despite of the above evidence, pancreatic cancer patients were found to have reduced total energy expenditure (TEE) due to a reduction in their physical activity level (PAL) [54].

Recently, research on the link between brown adipose tissue (BAT) and cachexia has been brought to the surface. The history of this link goes back to the study which examined autopsy samples of peri-adrenal tissue that showed BAT to be present in 80% of cachectic cancer patients, compared with only 13% of age matched controls [55]. BAT has a well-established role in controlling body thermostasis and energy balance despite its scarcity in adult humans. Thermogenesis in BAT and its possible link to cachexia was studied in the context of REE. Thethermogenic effect of BAT and skeletal muscle is probably due to the function of uncoupling proteins (UCP). UCPs are mitochondrial inner membrane proteins that can dissipate the proton gradient before it can be used to provide the energy for oxidative phosphorylation by decreasing the level of coupling of respiration to ADP phosphorylation [56]. There are five types of UCPs: UCP1 found only in BAT, UCP2 found in most tissues, UCP3 found only in BAT and skeletal muscle, UCP4 found in brain tissue, and UCP5 found in brain, testis and others [57].Of these five, UCP1 is considered to be most important, although UCP3 also plays an important role in energy balance and lipid metabolism. In a murine model of cachexia, mRNA expression levels of UCP1, but not UCP2 and 3, in BAT was significantly elevated over controls [58]. However, expression of UCP2 and 3 were significantly increased in skeletal muscle [58]. In cachectic cancer patients, UCP3 expression level in *rectus abdominis* muscle was five times higher than controls [59]. This increased expression level might be the mechanism by which REE is increased in skeletal muscles of cachectic patients. Therefore, the increase in REE is not merely the main cause of cachexia.

Another way of which energy balance can be a cause of cachexia is the "futile energy cycle". This futile cycle, a phenomenon termed "Warburg effect", is used by most cancer cells to generate ATP. Warburg effect utilized glycolysis followed by

lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria in most normal cells as the principal method to generate energy [60]. Malignant cells typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin even with the presence of oxygen.Several reasons are attributed to this phenomenon. However, regardless of the possible reasons, the Warburg effect results in a net yield of two ATP molecules as opposed to oxidative phosphorylation that yields a total of approximately 30-32 ATP molecules. Thus, the conversion of glucose into lactic acid is an energy-inefficient process, which means that growth of tumors requires 40 times more glucose than if it was fully oxidized through the tricarboxylic acid cycle. In order for the lactate to be recycled by liver, a net of 4 ATP molecules are needed. Hence, the Warburg effect results in a negative balance of 2 ATP molecules; which is another energy inefficient process [61]. In weight losing colorectal cancer patients, the rates of glucose production and recycling were significantly higher than that in controls [62]. This complete futile process account for an approximately additional loss of energy in cancer patients of 300 kcal/day [63]. Regardless of these evidence, fast progressing cachexia such that evident in pancreatic cancer patients, cannot be attributed merely to this futile cycle. Pancreatic tumors are relatively small in size and require a long period to develop which is disproportional to the degree and intensity of weight loss observed in these patients.

1.4.2 Anorexia and Cachexia

Anorexia, or loss of appetite, is one of the symptoms of cachexia and is common in cancer patients. Cancer patients report early satiety. This may be the result of many factors such as: the mechanical obstruction of the gastrointestinal tract which may

hinder the passage of food, the functional abnormalities of the gastrointestinal mucosa resulting in malabsorption, or chemical triggers that suppresses appetite center in the brain. If anorexia, regardless of the cause, is the main reason of weight loss in cancer patients, one should expect a direct correlation between reduced food intake and the degree of weight loss. Surprisingly, the opposite is true! A large study of 297 cancer patients found that weight loss could not be accounted for by a diminished dietary intake, since the absolute amounts of energy intake did not differ, and the intake per kilogram of body weight was actually higher in the weight-losing patients compared with the weight-stable patients [64].

Also, if anorexia is a direct and essential etiology for cachexia, nutritional supplementation to compensate for the deficiency in caloric intake would be expected to promptly reverse cachectic state. However, clinical trials showed that the wasting process in cancer patients cannot be reversed by all means of nutritional supplementation such as dietary counseling [65], total parenteral nutrition [66], or appetite stimulants such as cryoheptadine [67], or dronabinol. In addition, weight gain due to the total parenteral nutrition was transient and translated into an increase in fat and water rather than lean body mass [66]. This effect is not only limited to weight loss in cancer patients, but was also observed in patients with human immunodeficiency virus [68], and sepsis [69]. However, nutritional supplementation in pancreatic cancer patients has some survival advantage despite of its inability to reverse body weight loss [70]. These results show that anorexia is an important symptom of cachexia but is not the sole suspect for the loss of body mass.

1.4.3 Cachexia as a Catabolic State

In the absence of stimuli, adult muscle mass remains constant with relatively equal rates of protein synthesis and degradation. However, an imbalance via depression in protein synthesis, an increase in protein degradation, or a combination of both should occur for cachexia to develop; research evidence is available for all the possibilities. For example, one study suggested that cachexia is primarily a result of alterations in protein synthesis rather than protein degradation [71]. Other study suggested the high protein turnover rates in patients with hepatocellular carcinoma to be the result of an elevated rate of protein breakdown, with oxidation of the released amino acids [72]. However, the majority of studies suggest that both processes are occurring simultaneously. There are three major proteolytic pathways responsible for the degradation of proteins in skeletal muscle; the lysosomal system, the calcium-activated system, and the ubiquitin-proteasome pathway [73].

The lysosomal system includes the acid optimal cysteine proteases cathepsins B, H, and L as well as the aspartate protease cathepsin D. This system is mainly responsible for the degradation of extracellular proteins, cell receptors, endocytosed proteins, and phagocytosed bacteria rather than influencing the normal turnover of cytosolic proteins [74, 75]. This degradation system is accelerated by glucagon in the liver and the lack of insulin or essential amino acids [76]. The second degradation system is the ATP-independent, calcium-activated, cytosolic cysteine proteases system that are activated by an increase in cytosolic calcium which is mainly involved in tissue injury, necrosis, and autolysis. These proteases include calpains I and II [77-79]. The third degradation system is the ATP- and ubiquitin-dependent proteasome pathway. The function of the ubiquitin-proteasome pathway is to degrade defective protein products produced from errors in translation or from

oxidative stress [80, 81]. It works in harmony with the calpain system to disassemble and degrade muscle myofilaments [82]. The defective protein is targeted by the addition of multiple ubiquitin molecules and the subsequent recognition and degradation by the 26S proteasome [83]. Polyubiquitination of the protein is a process that not only tag proteins for degradation, but also ensures the specificity of the process that is normally handled by the proteasome. For polyubiquitination to occur, ubiquitin is activated by an activating enzyme (E1) in two steps. Firstly, an intermediate is formed by ATP hydrolysis connecting adenosine monophosphate (AMP) with the carboxy-terminal carboxyl group of glycine in ubiquitin. Secondly, a thioester linkage with a cysteine residue in E is formed [81]. Then, the ubiquitin carrier protein (E2) accepts this ubiquitin to its active site at a cysteine residue. This is followed by E2 carrier protein recognizing the ubiquitin protein ligase (E3). Then, the E3 ligase transfers ubiquitin from the E2 thioester intermediate either to a specific ubiquitin binding site or to an isopeptide linkage with some degree of substrate specificity [73]. After multiple rounds of E3, ubiquitin ligation creates a polyubiquitin chain on the target protein. Once the proteins are marked with a polyubiquitin chain, they are degraded into oligopeptides (peptides containing six to nine amino acid residues) by the 26S proteasome [84]. These are then degraded by the giant protease tripeptidyl peptidase II (TPPII) and various aminopeptidases. TPPII cleaves peptides generated by the proteasome into tripeptides [85]. In normal situations, this process is not rate-limiting for proteolysis. However, it is important because the accumulation of abnormal peptides may be injurious to the cell.

1.4.3.1 Ubiquitin Proteasome Pathway System and Cachexia

The ubiquitin proteasome pathway system is responsible for the majority of skeletal muscle protein catabolism [73] and has been extensively reviewed [86]. This pathway is activated in catabolic states resulting in muscle atrophy. Thus, it more

likely accounts for the advanced proteolysis seen in wasting conditions such as fasting, sepsis, metabolic acidosis, acute diabetes, weightlessness and cancer cachexia [87].

In vitro studies of atrophying muscles have demonstrated no involvement of the lysosomal proteases or calcium-activated proteases systems. Yet, inhibiting ATP production decreased the rate of proteolysis to that of control muscles indicating that the ATP-dependent ubiquitin proteasome pathway is primarily responsible for skeletal muscle degradation [88, 89].

In mice bearing the cachexia-inducing MAC16 tumor, both proteasome proteolytic activity and TPPII activity increase in parallel with weight loss reaching a maximum at 16% weight loss, after which there was a progressive decrease in activity for both proteases with increasing weight loss [90]. In other animal models of cancer cachexia and in cancer patients, the ubiquitin proteasome pathway found to play the predominant role in the degradation of myofibrillar proteins especially in patients with >10% weight loss [91]. In yoshida ascites hepatoma (YAH)-bearing rats, removal of calcium or blocking the calcium-dependent proteolytic system did not inhibit muscle protein degradation, while inhibition of the lysosomal function reduced proteolysis by 12% in muscles [92]. Nevertheless, when ATP production was inhibited, proteolysis in muscles was similar to that of controls [92]. The same study showed a significant increase in ubiquitin mRNA (590-880%), the level of ubiquitin-conjugated proteins, and of mRNA for multiple proteosome subunits (100-215%) [92]. These findings indicate that the ubiquitin pathway is the predominant degradation system in animal models of cachexia.

Normally, the majority of cellular proteins are degraded by the proteasome pathway. However, for patients with a low weight loss (i.e. 2.9%), muscle biopsies showed no

change in components of the ubiquitin proteasome pathway, but rather an increase in the expression of cathepsin B mRNA [93]. Another study also showed an increase in lysosomal activity, as measured by cathepsin D and acid phosphatase, in skeletal muscle of cancer patients which in part of the subjects appears to correlate with weight loss [94]. Myofibrillar protein constitutes half of the total muscle protein and is lost at a faster rate than other proteins during atrophy. In a murine model of cachexia, myosin heavy chain was selectively targeted by the ubiquitin proteasome pathway in the cachectic state, while other core myofibrillar proteins including troponin T, tropomyosin (α- and β-forms), and α-sarcomeric actin remain unchanged [95].

With increasing evidence of the involvement of the ubiquitin proteasome pathway in regulating the skeletal muscle degradation in cancer cachexia, it is important to understand its triggers and how it gets activated.

1.4.4 Tumor and Host Factors Influencing Muscle Mass in Cachexia

1.4.4.1 Tumor Necrosis Factor-α

Animal studies provide a considerable evidence for the importance of tumor necrosis factor-α (TNF-α) in triggering muscle wasting in cancer cachexia. Transplantation of Chinese hamster ovary cells that is transfected with the human TNF-α gene in mice resulted in a syndrome resembling cachexia, with progressive wasting, anorexia, and early death [96]. Also, genetically engineered mice deficient in the TNF-α receptor protein type I which are transplanted with Lewis lung carcinoma showed reduced wasting of skeletal muscle compared with wild-type mice, despite the equal levels of serum TNF-α in both groups [97]. In addition, acute treatment of rats with recombinant TNF-α enhanced protein degradation and decreased protein synthesis in soleus muscle [98]. The mechanism of which TNF-α-

induced muscle wasting has also been studied. TNF-α has been shown to induce oxidative stress and nitric oxide synthase in murine skeletal muscle causing decrease in body weight, muscle wasting, and skeletal muscle molecular abnormalities, all of which are prevented by treatment with antioxidants or nitric oxide synthase inhibitors [99].

In humans, recombinant human TNF-α administered to patients as part of an antineoplastic regimen, caused a dose-related metabolic effects of enhanced energy expenditure with elevated CO_2 production, increased protein catabolism, peripheral efflux of amino acids, decreased total arterial amino acid levels, an increase in plasma cortisol level, elevated serum triglycerides, as well as increased glycerol and free fatty acid turnover [100]. These metabolic effects (both; muscle and fat) resemble those seen in refractory cancer cachexia, although its role in the human condition is questionable.

1.4.4.2 Interleukin-6

IL-6 decreased the half-life of long-lived proteins in *in vitro* studies in murine myotubes by increasing the activity of the proteasome, together with cathepsins B and L [101]. However, most research on IL-6 in cancer cachexia is in *in vivo* models with variable results. Inhibition of IL-6 secretion and binding to its cellular receptor by suramin, reduces the catabolism seen in colon-26 (C26) adenocarcinoma-bearing mice [102]. Also, muscle atrophy is seen in IL-6 transgenic mice that overexpress IL-6, which is completely reversed by IL-6 receptor antibody and is associated with increased mRNA levels for cathepsins (B and L) and ubiquitins [103]. On the other hand, wasting was not induced by administration of recombinant IL-6 in mice, even with repeated administration [104]. In contrast, using an $Apc^{Min/+}$ mouse, an established model of colorectal cancer and cachexia, to determine the role of

circulating IL-6 on the development of cachexia. Mice with the highest circulating IL-6 levels had the most severe cachectic symptoms [105]. Similarly, administration of IL-6 to rats acutely activated both total and myofibrillar protein degradation in muscle [106]. Unlike TNF-α, however, IL-6 produced no change in the expression of ubiquitin when administered intravenously to rats [107]. Thus, further research is needed to verify the role of IL-6 in cancer cachexia.

1.4.4.3 Interleukin-1ß

The data for the role of interleukin-1ß (IL-1ß) in cancer cachexia is controversial. Alike TNF-α, IL-1 can induce body weight loss and anorexia in mice [108]. Also, chronic treatment of rats with recombinant IL-1ß resulted in redistribution in body protein and a significant decrease in muscle protein content associated with a coordinated decrease in muscle mRNA levels of myofibrillar proteins [109].

1.4.4.4 Interferon-γ

In mice bearing Lewis lung tumors, administration of anti-interferon-γ (IFN-γ) monoclonal antibodies markedly decreased cachexia [110]. Also, nude mice inoculated with CHO-IFN-γ cells exhibited severe cachexia that was prevented in mice given anti-INF-γ monoclonal antibodies prior to injection of tumor cells [110]. Expression of ubiquitin gene in rat skeletal muscle was also up-regulated by IFN-γ in a similar manner to TNF-α and IL-1ß [107].

Studies have investigated individual key players in triggering the main degradation pathway in cancer cachexia (i.e. ubiquitin proteasome pathway), but also it looked at the effect of combining these elements together. For example, myotubes and mouse muscles treated with TNF-α together with IFN-γ resulted in a significant reduction in myosin expression through an RNA-dependent mechanism which indicates that these two cytokines are complementary in the progression of muscle degradation

[95]. Nevertheless, murine colon-26 adenocarcinoma treated with antibodies to IL-6, but not TNF-α or IFN-γ, attenuated the development of weight loss and other key parameters of cachexia [111]. However, serum levels of cytokines, including IFN-γ, TNF-α, IL-1ß, and IL-6 are poorly correlated, so far, with weight loss and cachexia in cancer patients [112].

1.5 Gene Expression Profiling as a mean of Understanding Pathology

The transcriptome is the total set of RNA molecules (i.e. transcripts) and their quantity in a given cell, tissue, organ, or a whole organism [113]. Unlike genome, which is relatively static, transcriptome varies from place to place and time to time in response to developmental, environmental, or pathological stimuli [114]. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease [113, 114]. One way for the characterization of a physiologic and/or pathological condition in a given tissue can be determined by its differentially active genes [114]. "Gene expression profiling" is a molecular biology technique that measures and reflects on the activity of many to all of the expressed genes between two (pairwise comparison) or more sample types (i.e. case/s vs. control/s) at a given time.

After passing the era of "Human Genome Project", it was necessary to bridge the gap between gene sequence and function. A novel technology called "DNA Microarray" was introduced which enabled simultaneous measurement or a whole transcriptome analysis of the activity of virtually all genes in the genome. Whole transcriptome analysis can be done in two main methods; the hybridization- or the sequence-based approaches. The hybridization-based approach utilizes differentially fluorescently labeled cDNA molecules from the desired sample versus

its control. The mixed equal quantities of cDNA are then incubated on a microarray plats that contain spots with probes targeting the desired genes. Gene activity based on microarray technology relies on spots brightness captured through these probes that hybridize to the differentially fluorescently labeled groups of samples. The intensity of brightness varies from gene to gene and correlates to the transcriptional activity of the examined genes. This technology was an enabler in many aspects of cancer biology; we can differentiate between tumor and non-tumor, different tumor grades, risk of tumor metastasis, etc. It even enabled clinicians make decisions about the optimal treatment of cancer patients. However, the technology did not stop there! Thus, understanding the transcriptome plays a pivotal role in interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease.

The sequence-based approaches uses high-throughput next generation sequencing techniques that were developed based on DNA microarray technology. The use of next-generation sequencing technology to study the transcriptome at the nucleotide level is known as RNA Sequencing (RNA-Seq). RNA-Seq, also called Whole Transcriptome Shotgun Sequencing [115], is a technology that uses next-generation sequencing capabilities to reveal an overview of RNA expression and quantity from a genome at a given moment in time [116].

Despite being a yet evolving technology that is yet under active development, RNA-Seq is expected to revolutionize our understanding of science [113]. It is a technology that outweighs all its counterparts in its advantages. Yet, a major exclusive setback is the bioinformatics challenges in assuring the righteous and in managing these tremendous representations of data [113].

Chapter 2: Research Aim, Hypothesis, and Specific Objectives

Given the fact that cancer cachexia has been known for such a long time, our understanding of the mechanisms of cancer cachexia lag way behind our knowledge of other aspects of tumor biology. This is despite the increase of the prevalence and significance of the syndrome. Exploratory clinical research into the basic etiopathological causes of cancer cachexia is lacking and we don't currently have any approved effective treatment for the involuntary weight loss in cancer patients.

The main aim of this thesis was to explore early differential changes in gene expression in cancer patients with or without cachexia using state-of-the-art whole expression profiling technology (RNA-Seq) in order to identify the mechanistic pathway/s responsible for early development of cancer-associated cachexia. This can bring reliable, representable, and consistent data from the clinic and back to the bench with more focused insights to be investigated and verified.

It is our hypothesis that identification of early changes in gene expression using whole transcriptome analysis in cachexia will lead to an improved understanding of the triggering mechanism in cancer patients. Also, understanding cancer cachexia can be best achieved through studying representative clinical muscle and fat samples.

Our research specific objectives were to:

1. Collect representative muscle and fat biopsies from cancer patients with cachexia and match them with non-cachectic weight-stable cancer controls.
2. Conduct global gene expression assays from high quality representative muscle and fat biopsies from cancer patients with early cachexia and compare them to non-cachectic weight-stable cancer controls.

3. Identify differential up and downregulated pathways involved in each of the two tissues.
4. Identify and analyze the highly significantly up and downregulated altered genes in each of the two tissues.
5. Verify expression data from clinical and preclinical models of cancer cachexia against the global gene expression results.
6. Identify and analyze the overlapping differentially expressed genes between cachectic skeletal muscles and cachectic adipose tissues.
7. Confirm increased or decreased expression of a selected genes using real-time RT-PCR.

Chapter 3: Materials and Methods

3.1 Ethical Approval

The ethical approval request was submitted to "Al Ain Medical District Human Research Ethics Committee (AAMDHREC)" at the College of Medicine and Health Sciences (CMHS) in the United Arab Emirates University (UAEU). AAMDHREC is accredited by the Federal Wide Assurance (FWA) under the number 00007109. Research ethical proposal and protocol obtained under approval number AAMDHREC 11/49. Written informed consents were obtained from each subject and each subject was also given “Patient Information Sheet” in the language of preference (i.e. Arabic or English) as per the committee recommendation.

3.2 Subjects

A total of 24 biopsies were obtained; 12 skeletal muscles and 12 visceral adipose tissue. Each set of the two tissues contains 6 biopsies from cachectic cancer cases and 6 weight-stable controls. All subjects (cachectic and weight-stable) were with diagnosed malignancies. All samples were obtained through elective surgeries. Inclusion and exclusion criteria were as following:

Inclusion Criteria: Cancer patients of any race, sex, and age equal or above 21 year-old with histologically proven malignancy and under active treatment. All cases should have documented or self-reported weight-loss (cachexia) that is measured by 5-10% involuntary weight loss of the total body mass in the past 3-6 months who are undergoing elective abdominal surgery who agreed to sign an informed consent.

Exclusion criteria: Subjects who are under 21 year-old. Any subject with any autoimmune and/or muscular disease, uncontrolled diabetes or thyroid disorder,

history of treatment with any anabolic/catabolic agents for the past 6 months, or history of physical impairment that affects mobility was excluded.

Control subjects were matched for age and diagnosis (as possible) and with no documented or self-reported weight loss or gain in the past 3-6 months prior to surgery (see Figure 1 for study flow diagram).

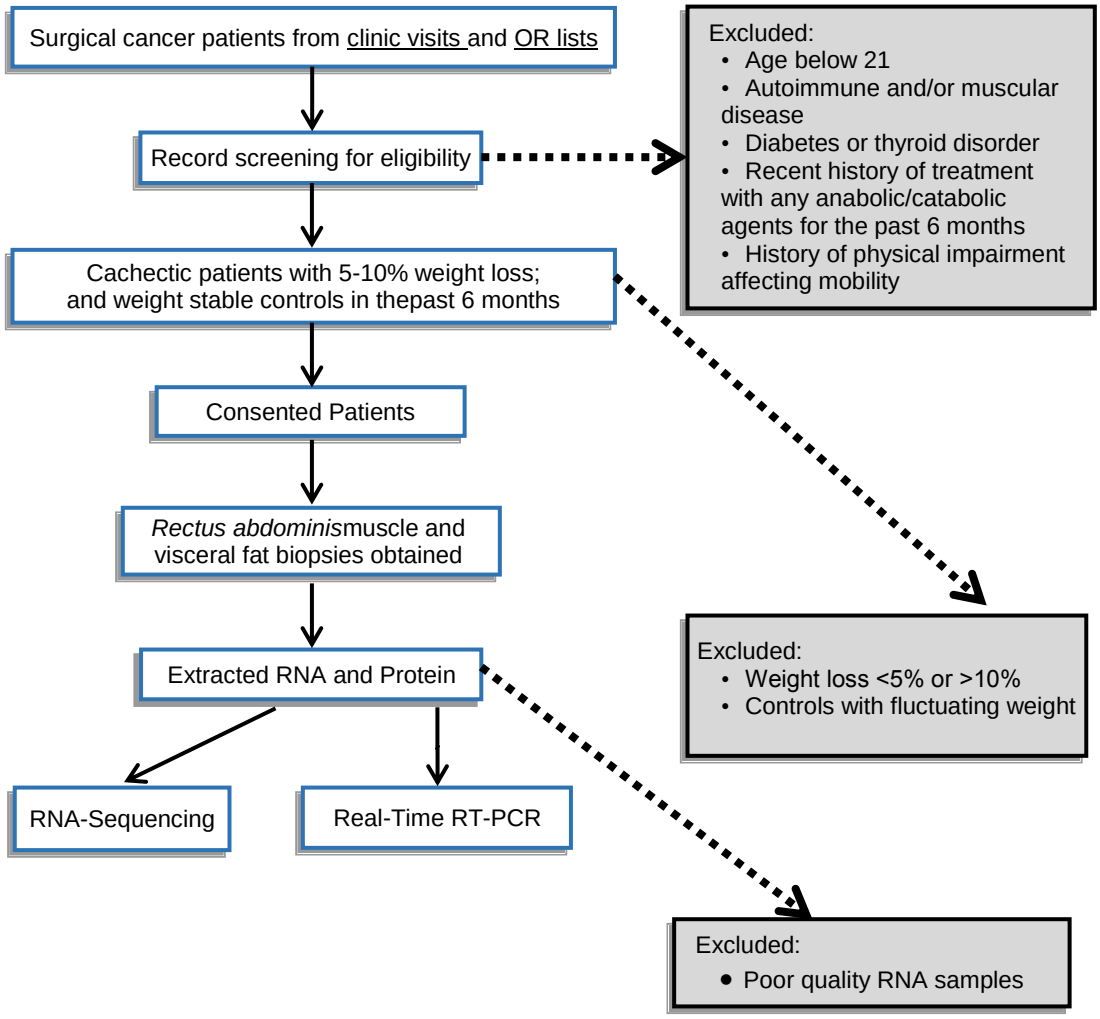

Figure 1: Study Flow Diagram.

3.3 Clinical Parameters

Body weights were measured with participants in light clothing using a beam scale (Seca). Heights were measured using a standard wall mounted measure and body mass indices (BMI) were calculated. All current available nutritional and inflammatory clinical parameters were recorded. History of weight changes from patients' records and/or self-reported weights were also recorded.

3.4 Collection of Muscle and Adipose Tissue Biopsies

All biopsies were taken at the start of either open or laparoscopic abdominal surgeries. Specimens of approximately 0.5-1.0 cm^3 from the rectus abdominis and/or visceral fat were removed. The biopsies were washed with normal saline solution, blotted, and immediately placed in RNALater (AM7021; Applied Biosystems, Carlsbad, CA, USA) and kept overnight at 4ºC to allow thorough penetration of the tissue. Tissue samples were then frozen at –80ºC until further processing.

3.5 Sample Labeling and Subject Confidentiality

Collected samples were given identification labeling composing of two letters to denote origin (i.e. "M" for muscle, and "F" for fat) and group (i.e. "S" for cachexia cases, and "C" for weight-stable controls). Each sample is then followed by a unique identification number. To ensure confidentiality, all other subject identifications were removed and never communicated in downstream sample processing. For future possible reference to subject data, the link of these labeling and actual medical record numbers is kept under the access of the leading experimenter and the principle investigator only.

3.6 Samples Preparation for RNA and Protein Extraction

Samples of muscle (45-59 mg) adipose tissue (95-173 mg) were homogenized in ice in 1.2 ml of trizol Reagent (15596-026, TRIzol® Reagent, Life Technologies, Thermo Fisher Scientific, USA) using the polytron homogenizer (POLYTRON® PT 2100 Homogenizers, Kinematica, Switzerland). Sample homogenizations were done at intermittent intervals with samples kept in ice in between to avoid RNA and protein degradation. Intermittent homogenization was the best method to ensure complete retrieval of RNA and protein from samples that are difficult to homogenize (e.g. muscle) or those who have low RNA and protein yield (i.e. fat) [117-119]. Homogenization was done in batches of 6 samples or less at 1500-2000 rpm. Completion of each batch took 3 hours (fat) and 4-5 hours (muscle) till no clear visible homogenate particles were observed. Polytron generator was cleaned in between samples in the following sequence; DNase/RNase free distilled water, 70% ethanol in DNase/RNase free distilled water, followed by DNase/RNase free distilled water. To ensure proper removal of tissue sticking on the generator, a solution of 1% of sodium dodecyl sulfate in DNase/RNase free distilled water was used every 1-2 hours and as needed to clean the probe.

3.6.1 RNA Extraction

Once a complete tissue homogenization had been achieved, RNA extraction was carried out using a combination of the liquid-liquid extraction technique; the acid guanidiniumthiocyanate-phenol-chloroform extraction (AGPC) [120, 121], and the column-based system. The AGPC method was used to separate the aqueous phase (RNA containing phase). This step was preceded with an additional centrifugation for fat samples *only* in order to remove the floating fat layer before the addition of the chloroform. The total RNA was then precipitated using ethanol and processing of

RNA isolation was completed using the SV Total RNA Isolation System® (Promega, Madison, USA) according to manufacturer's instructions. The concentration and purity of the RNA samples were determined by measuring the absorbance at 260 nm and the ratio of the absorbance at 260/230 and 260/280 nm using spectrophotometer (ND-1000 NanoDrop, Thermo Scientific, Wilmington, DE, USA). Only ratios ≥ 1.8 were accepted or else, extractions were repeated till the required purity was achieved. RNA samples were kept on ice during processing and quality check and then stored at -80º C pending further processing.

3.6.1.1 RNA Concentration

Sample yields below 20 ng/µl were concentrated using a cold vacuum centrifuge. This was followed by another check for RNA concentration and purity using spectrophotometer (ND-1000 NanoDrop, Thermo Scientific, Wilmington, DE, USA).

3.7 Reverse Transcription

Total RNA was converted into cDNA using a High Capacity cDNA Reverse Transcription Kit® (High Capacity cDNA Reverse Transcription Kit; 4374966; Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (420ng) was converted into cDNA in a 30µl reaction volume using the following reaction (Table 1) and was repeated whenever needed:

Table 1: Reverse Transcription Reaction.

Reverse Transcription Master Mix Components	**Volume (1x)**
10 x RT Buffer	3.0 µl
25 x dNTP Mix	1.2 µl
10 x RT Random Primers	3.0 µl
Multiscribe™ Reverse Transcriptase	1.0 µl
RNASE Inhibitor	1.0 µl
RNase Free H_2O	= (30 - 9.2 - vol of 420ng template) µl
Total Volume Reaction	30.0 µl

The above reaction gives a final cDNA concentration of = 420ng/30µl = 14ng/µl. Each cDNA sample were then diluted to 4ng/µl and stored in -20º C. The reverse transcription reaction was carried out in a Veriti thermal cycler (Life Technologies, Applied Biosystems, USA) using the following parameter values (Table 2):

Table 2: Parameters of the Thermal Cycler for Reverse Transcription.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temp ºC	25 ºC	37 ºC	85 ºC	4 ºC
Time	10 minutes	120 minutes	5 minutes	∞

3.8 Real-Time Reverse Transcription Polymerase Chain Reaction

The expression levels of mRNA were analyzed using target specific TaqMan® gene expression assays using real-time reverse transcription polymerase chain reaction (RT–PCR) and performed in a 7900HT Fast ABI Prism 7900HT Sequence Detection System (Applied Biosystems, USA). The following TaqMan® primers and probes kits were purchased from Applied Biosystems (Table 3):

Table 3: List of the TaqMan® Primers for Real-Time RT-PCR.

Assay ID	**Gene Symbol**	
Assay ID: Hs01098873_m1	Gene Symbol: COL4A2, hCG33042	
Assay ID: Hs00373339_m1	Gene Symbol: NRXN2, hCG1810991	
Assay ID: Hs00394748_m1	Gene Symbol: AGRN	
Assay ID: Hs01062014_m1	Gene Symbol: NOTCH1, hCG1818285	
Assay ID: Hs00373136_m1	Gene Symbol: PTPRR, hCG25410	
Assay ID: Hs00192297_m1	Gene Symbol: NDUFS1, hCG17250	
Assay ID: Hs00174877_m1	Gene Symbol: LEP, hCG33000	
Assay ID: Hs00223332_m1	Gene Symbol: TNMD, hCG20191	
Assay ID: Hs00896336_m1	Gene Symbol: LHCGR, hCG16776	
Assay ID: Hs04187682_g1	Gene Symbol: CXCL11, hCG23841	
Assay ID: Hs02800695_m1, #4326321E	Gene Symbol: HPRT1	Endogenous Controls
Assay ID: Hs02758991_g1, #4326317E	Gene Symbol: GAPDH	
Assay ID: Hs01060665_g1, #4326315E	Gene Symbol: ACTB	

The reaction mix of 10µl containing a total cDNA of 4ng/reaction was prepared using TaqMan® H Fast Universal PCR Master Mix, No AmpErase H UNG (Life Technologies #4352042, Applied Biosystems, USA) as follow (Table 4):

Table 4: Real-Time RT-PCR Reaction.

Real Time PCR Master Mix Reaction	**Volume (1x)**
DNase/RNase free H_2O	3.5 µl
cDNA (stock concentration 4ng/µl)	1 µl
2 X TaqMan H Fast Universal PCR Master Mix	5 µl
20 X Gene of Interest (GOI)	0.5 µl

Quantitative real-time PCR assay for the gene of interest was performed in duplicates and in a singleplex PCR reaction. The PCR thermal cycling parameters were run in fast mode as follow (Table 5):

Table 5: Parameters of the Thermal Cycler for Real-Time RT-PCR.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temp ºC	50º C	95 º C	40 cycles of 95 º C	60 º C
Time	2 minutes	20 seconds	1 second	20 seconds

Results were initially analyzed with the ABI Prism 7900HT SDS program v2, 4, all remaining calculations and statistical analysis were performed by the SDS RQ Manager 1.1,4 software using the $2^{-\Delta\Delta Ct}$ method with a relative quantification RQmin/RQmax confidence set at 95%. Three different endogenous controls were tested; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human Hypoxanthine phosphoribosyltransferase (Hprt1) rRNA, and human β-actin. Hprt1 was found to be the best and used to normalize expression results. Other expression related guidelines were followed from the MIQE guidelines [122].

3.9 Statistical Analysis

Preliminary statistical analysis of the next generation sequencing was carried out by our collaborator in Qatar. Confirmation of changes in gene expression by real-time RT-PCR was done using Student's t-test and analysis was done using the GraphPad Prism Software (version 5).

3.10 Bioinformatics Tools

Much freely available databases and software were used check gene functions, expression pattern, protein interaction, and functional pathways. These databases and software are:

- The US National Library of Medicine National Institutes of Health: http://www.ncbi.nlm.nih.gov/pubmed
- UCSC Genome Bioinformatics: http://genome.ucsc.edu/
- Ensembl: http://www.ensembl.org/index.html
- KEGG database: http://www.genome.jp/kegg/kegg1.html
- String: http://string-db.org/
- Biological General Repository for Interaction Datasets: http://thebiogrid.org/
- ExPASy Bioinformatics Resource Portal: http://www.expasy.org/proteomics/protein-protein_interaction

3.11 RNA Sequencing

Muscle and fat RNA samples (600ng) were sent in dry ice to our collaborators in Weill Cornel Medical College in Qatar for RNA Sequencing (RNA-Seq). RNA-Seq is a revolutionary transcriptomic technique that uses deep-sequencing technologies. The technique utilizes a population of RNA that is reversed transcribed into a library of cDNA fragments.

All samples underwent quality check prior to sequencing by measuring the RNA Integrity Number and ensuring that all samples are with a RNA Integrity Number value of at least 7. Samples were then reversed transcribed into cDNA. RNA-Seq was then performed on the Illumina HiSeq 2500 System.

Chapter 4: Results and Discussions – Muscle

4.1 Demographic and Biochemical Characteristics of Subjects

4.1.1 Results

Demographic and biochemical details of patients for muscle cachexia cases (MS) and muscle weight-stable controls (MC) are shown in bellow tables. The table shows the data after excluding two samples (one of each group) since they were outliers from the RNA-Seq data. The cause of these two samples as outliers was investigated. Data on the excluded cachexia sample was obtained through patients self-report of weight changes in the past six months with the complete absence of baseline data in patient's record. The patient was a referral to the hospital from a different city just to be operated. On the other hand, further investigation of the excluded control sample in patient's extended electronic records in different health institutions found to have obvious fluctuation of patients weight in between the two time points that our study has initially selected patients on. All samples were then analyzed based on weight, BMI, some nutritional parameters, inflammatory markers, and other related demographics.

Table 6 shows subjects' oncological diagnoses for muscle biopsies. All patients were with histologically confirmed malignancies. Age and gender distribution were not significantly different between cachexia muscle cases (MS) and weight-stable muscle controls (MC) (Table 7). All biochemical nutritional parameters (i.e. hemoglobin, total protein, albumin, urea, and creatinine) and inflammatory markers (i.e. WBC and CRP) were not significantly different between both groups (Table 7). Baseline weights and BMIs in the cachexia group did not differ significantly from the control. The time range between current (at time of biopsy) and baseline weights

(preceding date of biopsy) was 3-6 months. Mean weight loss from baseline in the cachexia group was 4.6 kg which is 6.5% of baseline body weight (i.e. 3-6 months before the date of sample collection). Amount and percentage of weight loss was significantly different from controls that had stable weights; P-value of 0.0014 and 0.0003, respectively.

Table 6: Subjects' Diagnoses for Skeletal Muscle Biopsies.

Cachectic Muscle Cases (MS)	**Weight-stable Muscle Controls (MC)**
MS1: Malignant Neoplasm of Rectum	MC1: Malignant Neoplasm of Rectum
MS2: Malignant Neoplasm of Stomach	MC2: Malignant Neoplasm of Rectum
MS3: Malignant Neoplasm of Rectum	MC3: Malignant Neoplasm of Colon
MS4:Malignant Neoplasm of Duodenum	MC5: Malignant Neoplasm of Connective and Soft Tissue
MS6: Malignant Neoplasm of Pancreas	MC8: Malignant Neoplasm of Colon

Table 7: Demographic Data for Cachectic Muscle Cases and Weight-Stable Muscle Controls.

Gender (M/F) MS: 4/1; MC: 3/2 **Characteristics**	**MS: N=5 MC: N=5**	**Mean**	**SEM**	**Median**	**Range**	**P-Value**
Age	Cach. Cont.	61.40 53.40	7.461 6.447	67.00 59.00	44-83 36-66	0.4406
Hemoglobin	Cach. Cont.	114.0 114.6	4.669 8.583	111.0 122.0	103-125 90-135	0.9525
Creatinine	Cach. Cont.	65.75 61.60	10.23 3.385	69.00 63.00	41-84 52-72	0.6837
Urea	Cach. Cont.	6.650 3.480	1.735 0.3980	6.300 3.900	2.9-11.1 1.9-4.0	0.0863
Total protein	Cach. Cont.	52.75 51.60	4.535 3.516	49.50 49.00	46-66 42-63	0.8441
Albumin	Cach. Cont.	27.50 30.00	4.349 2.280	25.00 29.00	20-40 23-37	0.6047
WBC	Cach. Cont.	8.900 8.640	1.307 1.613	9.000 9.600	6.0-13.3 4.4-12.1	0.9034
CRP	Cach. Cont.	12.50 14.67	7.331 2.333	7.500 14.00	1-34 11-19	0.8172
Baseline BMI	Cach. Cont.	23.58 29.86	2.378 1.765	23.02 30.46	15.57-29.24; 25.26-35.38	0.0667
Current BMI	Cach. Cont.	21.95 29.79	2.030 1.811	21.98 30.46	14.81-26.44; 24.91-35.38	*0.0205
Baseline weights	Cach. Cont.	67.92 81.20	5.738 2.653	73.00 84.00	45.00-74.60; 73.00-87.00	0.0689
Current weights	Cach. Cont.	63.38 81.00	5.213 2.811	67.90 84.00	42.80-71.20; 72.00-87.00	*0.0177
Amount of weight loss	Cach. Cont.	4.540 0.2000	0.8818 0.2000	4.000 0.0000	2.200-7.000 0.0000-1.000	**0.0014
% of weight loss	Cach. Cont.	6.552 0.2740	0.9994 0.2740	5.479 0.0000	4.558-9.589 0.0000-1.370	***0.0003

Table 8 compares weight and BMI changes between current and baseline within each group (paired t-test). The cachectic muscle cases (MS) had a significant difference between current and baseline data while weight-stable controls (MC) had no significant change.

Table 8: Paired t-Tests for Weight and BMI Changes for Muscle Biopsies.

Characteristics	**N=MS/MC; 5/5**	**Mean**	**SEM**	**Median**	**Range**	**P-Value**
Weight MS (cachectic)	Baseline Current	67.92 63.38	5.738 5.213	73.00 67.90	45.00-74.60; 42.80-71.20	** 0.0068
BMI MS (cachectic)	Baseline Current	23.58 21.95	2.378 2.030	23.02 21.98	15.57-29.24; 14.81-26.44	* 0.0140
Weight MC (weight-stable)	Baseline Current	81.20 81.00	2.653 2.811	84.00 84.00	73.00-87.00 72.00-87.00	0.3739
BMI MC (weight-stable)	Baseline Current	29.86 29.79	1.765 1.811	30.46 30.46	25.26-35.38 24.91-35.38	0.3739

4.1.2 Discussion

In the above demographic analysis, patients included in the study were chosen meticulously to be at the early stages of cachexia (i.e. 5 - < 10% weight loss). Consensus definition had identified early cachexia as weight loss >5% or BMI <20kg/m^2 and weight loss >2% or sarcopenia and weight loss >2% with often reduced food intake or systemic inflammation [7]. Our subjects had a mean percentage of weight loss of 6.5% (range 4.6-9.6 %). Since systemic inflammation measured via serum CRP may or may not be present in patients with cachexia [7], our subjects were included regardless of their CRP values (range 1-34, Table 6). However, the CRP values between cachectic cases (MS) and weight-stable controls (FC) were ensured to be not statistically significant; P= 0.325 in muscle.

Nutritional and nutrition-related biochemical markers were all ensured to be comparable in both groups to objectively exclude the possibility of malnutrition as the primary cause of weight loss.

4.2 Heat Map of RNA Sequencing Data

4.2.1 Results

Figure2shows the heat map of differentially regulated genes between cachectic muscle cases (MS) and weight-stable controls (MC). The figure shows a complete and clear cut separation between both groups. The heat map shows a total of 238 significantly upregulated genes and 235 downregulated genes (P-value < 0.05; and FDR < 0.5).

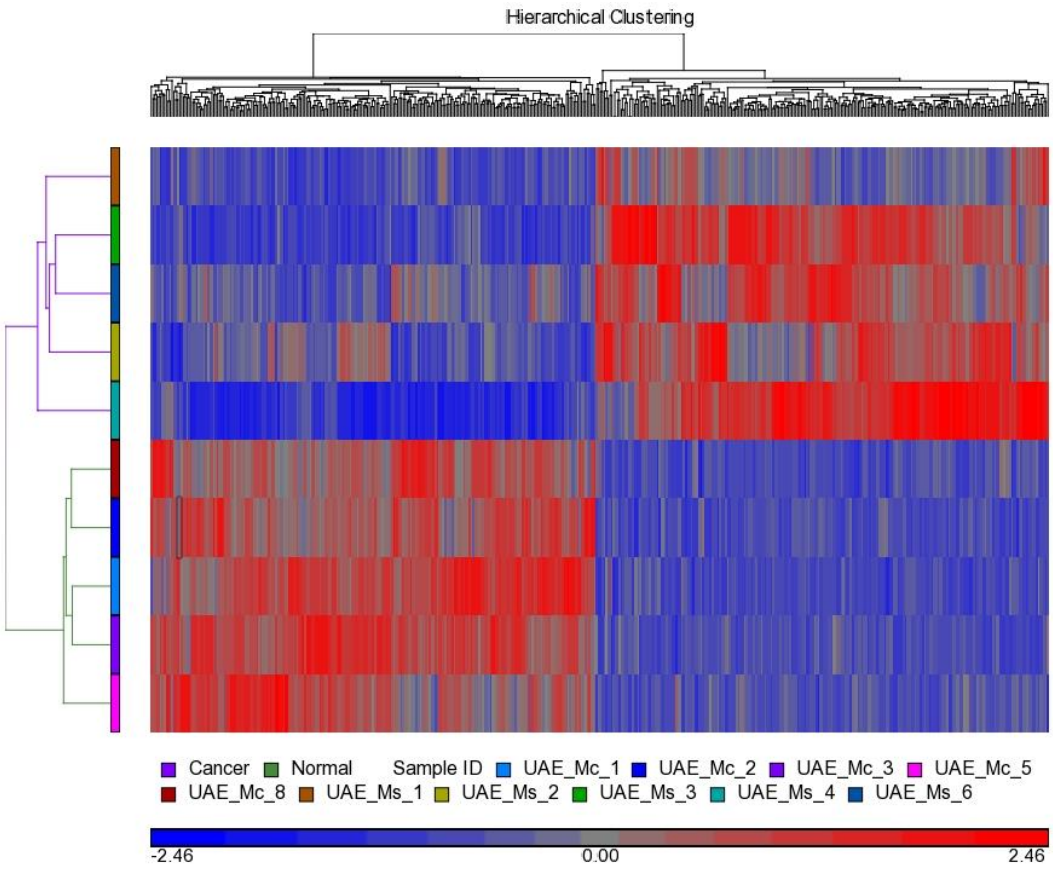

Figure 2: Heat Map Representation of the Differentially Expressed Genes in Skeletal Muscle Tissue.

Gene expression is shown for the cachectic muscle cases (MS; purple bars; upper panel) and weight-stable controls (MC; green bar; lower panel). Colors represent scaled and centered expression values; red represents higher expression, blue represents lower expression.

4.2.2 Discussion

The heat-map provides a graphical visualization of the matrix dataset by representing individual values with different colors. This necessity stems from the increasing data volumes generated by scientific studies. Effective data visualization enables understanding data at both broad and detailed levels. Recent progress in high-throughput techniques, such as RNA-Seq, has increased the demand for the visualization of multi-dimensional in addition to the numeric data [123]. This simple

hierarchical cluster analysis revealed a clear visual distinction of gene expression signature between cachexia muscle cases (MS) from those with weight-stable controls (MC).

4.3 Analysis of Differentially Expressed KEGG Pathways

4.3.1 Introduction to Pathways Analysis

Even for the simplest biological function, single-gene expression analysis has its own limitations for understanding the biological mechanism of a disease/syndrome. Complicated biological and pathological processes are a function of hundreds of differentially expressed genes. These genes never work independently. Whole genome expression analysis draws attention for discovering the hidden links disregarded by the single-gene analysis.

The introduction of high throughput technology, like microarray, enabled simultaneous quantification of thousands of genes to the full genome expression. However, “next generation sequencing” is much more powerful than microarray in identifying a more quantitative expression of genes of the whole transcriptome from cells or tissues.

4.3.1.1 Why Pathways Analysis?

Pathways analysis has emerged as one of the most important methods to gain insight into the underlying biology of differentially expressed genes. It is a logical next step in any high-throughput experimentation. Basing the massive quantity of data generated through full genome expression assays on pathways analysis reduces the complexity of single gene analysis. Also, high-throughput experiments do not produce biological findings. Since gene products do not work alone, but in an intricate network of interactions, pathways analysis helps interpret the data in the

context of biological processes; pathways and networks. Thus, it gives a global perspective on the data and the problem in hand.

Pathways analysis also provides in-depth and contextualized findings to help understand the mechanisms of disease/conditions in question. It helps in identification of genes and proteins associated with the etiology of a specific disease. Also, it helps to understand how to intervene therapeutically in disease processes by prediction of drug targets. It aids in data integration from diverse biological information and hence the discovery of the function of unknown genes.

Many pathways analysis approaches and resulting software/tools are available. These tools have evolved in, so far, three generations of algorithms of increasing complexity and hence, comprehensiveness [124]. "KEGG pathway analysis" is one of the third generation tools that is widely used [124]. Tools in the first and the second generations consider only the number of genes in a pathway or gene coexpression to identify significantly altered pathways. Thus, they always produce the same results no matter how they are analyzed [124]. However, the third generation pathways analysis utilizes additional information available from these knowledge bases [124]. It is important to understand this concept during data analysis since variations in statistical significance is highly likely [125]. For example, statistical significance of cytokine-cytokine receptor interaction pathway calculated by three different methods resulted in P-values of 8.08×10^{-6}, 0.72, and 6.19×10^{-2} [125]. This huge difference should be always considered during data analysis. However, the real value of pathway analysis is to generate hypothesis, inferences, and explore the overall picture of a complex unknown diseases/conditions/syndromes.

4.3.1.2 KEGG Pathways

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database is a project that was initiated back in 1995 under the then ongoing Japanese Human Genome Program [126]. It is a collection of databases (seventeen main databases) that are broadly categorized into 4 broad categories that deals with genomes, biological pathways, diseases, drugs, and chemical substances. In our study, our collaborator in Qatar has initially utilized it to generate a computer representation from our molecular-level information (RNA Sequencing results) to explore differentially involved pathways in cachexia pathogenesis [126, 127]. The outcome of this analysis is the "KEGG pathway mapping", whereby the gene content in the genome is compared with the "KEGG PATHWAY" database to examine which pathways and associated functions are likely to be differentially encoded in the genome.

Several pathways were found to be differentially expressed; up- or downregulated, when expression data was analyzed using KEGG pathways analysis. Each of these pathways has several genes that are significantly altered and will be discussed below.

4.3.2 Upregulated KEGG Pathways in Cachectic Skeletal Muscle

4.3.2.1 Results

Four KEGG pathways are differentially upregulated in cachectic muscles cases vs. weight-stable control muscles; notch signaling pathway, cell adhesion molecules (CAMs), acute myeloid leukemia, extracellular matrix (ECM)-receptor interaction. These pathways have 4, 6, 4, and 4 significantly upregulated genes respectively (Table 9).Figure 2shows the individual genes in each pathway and the overall statistical significance for each of these pathways. Table 10 shows a summary of the full gene name, fold change, and P-value for individual genes in each pathway.

Table 9: Differentially Upregulated KEGG pathways in Cachectic Skeletal Muscle.

KEGG Pathway	**Gene**	**Gene**	**Gene**	**Gene**	**Gene**	**Gene**	**P-value**	**FDR**
Notch signaling pathway	NOTCH1	NOTCH4	MFNG	DTX2			0.021	0.87
Cell Adhesion molecules	F11R	CLDN11	ESAM	HLA-A	HLA-C	NRXN2	0.025	0.70
Acute myeloid leukemia	RARA	STAT5A	TCF7L2	RELA			0.036	0.70
ECM-receptor interaction	AGRN	COL4A2	HSPG2	VWF			0.088	0.90

Table 10: Data of Individual Genes in the Upregulated KEGG Pathways in Cachectic Skeletal Muscle.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Muscle-Up / Notch Signaling Pathway			
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	2.99	0.0022
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	5.69	0.0143
NOTCH4	Notch homolog 4 (Drosophila)	3.69	0.0040
DTX2	deltex homolog 2 (Drosophila)	3.70	0.0155
Muscle-Up / Cell adhesion molecules (CAMs)			
F11R	F11 receptor	2.59	0.0160
CLDN11	claudin 11	6.76	0.0158
ESAM	endothelial cell adhesion molecule	3.39	0.0004
HLA-A	major histocompatibility complex, class I, A	2.92	0.0162
HLA-C	major histocompatibility complex, class I, C; major histocompatibility complex, class I, B	3.56	0.0173
NRXN2	neurexin 2	11.83	0.0148
Muscle-Up / Acute myeloid leukemia			
RARA	retinoic acid receptor, alpha	3.06	0.0134
STAT5A	signal transducer and activator of transcription 5A	3.33	0.0150
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	2.36	0.0169
RELA	v-relreticuloendotheliosis viral oncogene homolog A (avian)	2.29	0.0069
Muscle-Up / ECM-receptor interaction			
AGRN	Agrin	2.75	0.0161
COL4A2	collagen, type IV, alpha 2	8.54	0.0184
HSPG2	heparan sulfate proteoglycan 2	7.79	0.0189
VWF	von Willebrand factor	3.22	0.0167

4.3.2.2 Discussion

Notch Signaling Pathway

Notch signaling is an evolutionarily conserved intercellular pathway that regulates interactions between physically adjacent cells and is a well-documented pathway in skeletal muscle development and regeneration [128]. Normally, Notch pathway in skeletal muscles regulates proliferation and commitment of activated satellite cells to myogenic lineage. Also, activation of Notch signaling is a prerequisite for the expansion of postnatal satellite cells and to prevent the premature differentiation of

myogenic progenitor cells precursors in injured myofibers into mature muscle [129, 130]. These two processes ensure muscular homeostasis. Muscle injury, which is the early cachexia in our case, is known to activate Notch pathway. Two known notch receptors NOTCH1 and NOTCH4 were upregulated. In addition, two known activators of the pathway [131, 132] were upregulated; MFNG, which encodes for manic fringe (a protein family that also includes the radical and lunatic fringe) which fucosylates and thereby modulates notch, and DTX2, which encodes for deltex-1 an E3 ubiquitin ligase which is positive regulator of downstream, were upregulated [133, 134].

Cell adhesion molecules and ECM-receptor interaction pathways

It is well-know that further activation of Notch pathway require more cell-cell contacts [135]. Thus, no wonder that two other supportive pathways; cell adhesion molecules (CAM), and extracellular matrix-receptor interaction, were also upregulated with multiple genes involved at different levels.

On a different note, elements in the extracellular matrix-receptor interaction pathway were also correlated to documented literature in muscle wasting. For example, agrin (AGRN) fragments are under investigation as a possible biomarker for sarcopenia [136] and was also found to be upregulated in cachectic muscles. Also, expression of collagen type 4 alpha 1 (COL4A1, from original data) and collagen type 4 alpha 2 (COL4A2) genes; which are components of the myocyte basal lamina [137], are significantly upregulated. The matrix metalloproteinase genes (MMP2 and MMP9) code for abundant gelatinases in muscle that hydrolyze fibrillar collagens [138]. The two genes which are normally expressed in quiescent satellite cells [139]. MMP2 is activated by MMP14 at the sarcolemma [138]. MMP2, its activator; MMP14 and MMP9 are massively upregulated (but not statistically significant) in the cachectic

muscles. This signature molecular changes in our cachectic muscles reflects the quiescent state of the satellite cells which are, possibly, at its borderline activation once the degrading forces for the type IV collagens (i.e. COL4A1 and 2) exceeds cellular degradation resistance threshold.

Acute myeloid leukemia pathway

Another upregulated pathway in cachectic muscles is the acute myeloid leukemia. One gene is of a particular and a justified importance in the cachexia context; the transcription factor 7-like 2 (TCF7L2) gene. This gene was found in a genome-wide association scans to be the most important locus predisposing and consistently involved in type 2 diabetes [140]. Insulin resistance is an important common feature between cancer cachexia and type 2 diabetes and patients with cancer cachexia have profound insulin resistance [141, 142].

A certain polymorphism that increases TCF7L2 gene expression is associated to type 2 diabetes [140] and it could be that the same gene involved in muscles contributing to insulin resistance in cachectic patients. Not only TCF7L2, but also another gene in the same pathway; the signal transducer and activator of transcription 5A (STAT5A) gene, plays an important role in insulin resistance. STAT5A is a physiological substrate of the insulin receptor that is independent of JAK [143]. Insulin activates STAT5A (along other stats) [143]. Prolonged insulin exposure in skeletal muscle cells increases STAT5 expression which may play an important role in the manifestation and/or progression of insulin resistance in muscles [144]. RELA, which is a member of the NFkappaB family, forms a heterodimer with p65-c-Rel to from a transcriptional activator. Expression of RELA gene, on the other hand, was increased in human and mouse model of Huntington's disease [145].

4.3.3 Downregulated KEGG Pathways in Cachectic Skeletal Muscle

4.3.3.1 Results

Eleven KEGG pathways are differentially downregulated in cachectic muscles cases vs. weight-stable muscles. Several of these pathways share many of the same genes (Table 11). We decided to collectively call these pathways (i.e. hypertrophic cardiomyopathy, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and cardiac muscle contraction); the cardiac muscle contraction and cardiac myopathy pathway. Also, via studying the nature of the genes involved in the insulin signaling pathway, we decided to call it glycogen metabolism pathway. Table 11 shows the individual genes in each pathway and the overall statistical significance for each of these pathways. Table 12 shows a summary of the full gene name, fold change, and P-value for individual genes in each pathway.

Table 11: Differentially Downregulated KEGG Pathways in Cachectic Skeletal Muscle.

KEGG Pathway	Gene	Gene	Gene	Gene	Gene	Gene	Gene	Gene	P-value	FDR
Hypertrophic cardio-myopathy	ATP2A2	CACNA2D1	CACNA2D3	ITGB6	SGCD	TTN	TPM2	PRKAB2	0.0001	0.012
Dilated cardio-myopathy	ATP2A2	CACNA2D1	CACNA2D3	ITGB6	SGCD	TTN	TPM2		0.001	0.063
Arrhythmo-genic right ventricular cardio-myopathy	ATP2A2	CACNA2D1	CACNA2D3	ITGB6	SGCD			CTNNA3	0.003	0.064
Cardiac muscle contraction	ATP2A2	CACNA2D1	CACNA2D3				TPM2	ATP1B4	0.02	0.078
Insulin signaling pathway	PHKA1	PHKB		PPP1R3A	PPP1R3B	PPP1R3C		PRKAB2	0.002	0.33
Valine, leucine and isoleucine degradation	AUH	ACADM	ACADSB	DLD					0.02	0.29
Parkinson's disease		NDUFB5	NDUFS1	ATP5B	PINK1	PARK2	UBE2G1		0.028	0.33
Calcium signaling pathway	ATP2A2	CAMK2G		PHKA1	PHKB	PLCB1	PPP3CB	PHKG1	0.029	0.31
Long-term potentiation		CAMK2G		RPS6KA3		PLCB1	PPP3CB		0.062	0.51
MAPK signaling pathway	EGF	CACNA2D1	CACNA2D3	RPS6KA3	SOS2	PTPRR	PPP3CB	MEF2C	0.064	0.48
Alzheimer's disease	ATP2A2	NDUFB5	NDUFS1	ATP5B	SOS2	PLCB1	PPP3CB		0.066	0.46

Table 12: Data of Individual Genes in the Downregulated KEGG Pathways in Cachectic Skeletal Muscle.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Muscle-Down / Hypertrophic cardiomyopathy (HCM)			
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-2.36	0.0096
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	-3.37	0.0018
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	-3.65	0.0052
ITGB6	integrin, beta 6	-5.09	0.0002
PRKAB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	-2.64	0.0001
SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	-2.53	0.0137
TTN	Titin	-2.09	0.0135
TPM2	tropomyosin 2 (beta)	-2.44	0.0019
Muscle-Down / Dilated cardiomyopathy			
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-2.36	0.0096
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	-3.37	0.0018
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	-3.65	0.0052
ITGB6	integrin, beta 6	-5.09	0.0002
SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	-2.53	0.0137
TTN	Titin	-2.09	0.0135
TPM2	tropomyosin 2 (beta)	-2.44	0.0019
Muscle-Down / Insulin signaling pathway			
PHKA1	phosphorylase kinase, alpha 1 pseudogene 1; phosphorylase kinase, alpha 1 (muscle)	-2.21	0.0185
PHKB	phosphorylase kinase, beta	-2.46	0.0016
PHKG1	phosphorylase kinase, gamma 1 (muscle)	-2.14	0.0142
PRKAB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	-2.64	0.0001
PPP1R3A	protein phosphatase 1, regulatory (inhibitor) subunit 3A	-2.52	0.0099
PPP1R3B	protein phosphatase 1, regulatory (inhibitor) subunit 3B	-2.97	0.0040
PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	-2.53	0.0081
SOS2	son of sevenless homolog 2 (Drosophila)	-2.39	0.0005
Muscle-Down / Arrhythmogenic right ventricular cardiomyopathy (ARVC)			
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-2.36	0.0096
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	-3.37	0.0018
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	-3.65	0.0052
CTNNA3	catenin (cadherin-associated protein), alpha 3	-4.38	0.0007
ITGB6	integrin, beta 6	-5.09	0.0002
SGCD	sarcoglycan, delta (35kDa dystrophin-associated	-2.53	0.0137

	glycoprotein)		
Muscle-Down / Cardiac muscle contraction			
ATP1B4	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	-2.87	0.0126
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-2.36	0.0096
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	-3.37	0.0018
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	-3.65	0.0052
TPM2	tropomyosin 2 (beta)	-2.44	0.0019
Muscle-Down / Valine, leucine and isoleucine degradation			
AUH	AU RNA binding protein/enoyl-Coenzyme A hydratase	-2.16	0.0007
ACADM	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	-3.64	0.0004
ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	-2.20	0.0055
DLD	dihydrolipoamide dehydrogenase	-2.09	0.0026
Muscle-Down / Parkinson's disease			
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	-2.10	0.0012
Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	-2.04	0.0009
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	-2.34	0.0005
Pink1	PTEN induced putative kinase 1	-2.16	0.0153
park2	Parkinson disease (autosomal recessive, juvenile) 2, parkin	-3.26	0.0012
ube2g1	ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast)	-2.01	0.0063
Muscle-Down / Calcium signaling pathway			
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-2.36	0.0096
CAMK2G	calcium/calmodulin-dependent protein kinase II gamma	-2.09	0.0068
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	-2.06	0.0057
PHKA1	phosphorylase kinase, alpha 1 pseudogene 1; phosphorylase kinase, alpha 1 (muscle)	-2.21	0.0185
PHKB	phosphorylase kinase, beta	-2.46	0.0016
PHKG1	phosphorylase kinase, gamma 1 (muscle)	-2.14	0.0142
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	-2.06	0.0139
Muscle-Down / Long-term potentiation			
CAMK2G	calcium/calmodulin-dependent protein kinase II gamma	-2.09	0.0068
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	-2.06	0.0057
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	-2.06	0.0139
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	-2.02	0.0001
Muscle-Down / MAPK signaling pathway			
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	-3.37	0.0018
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	-3.65	0.0052
EGF	epidermal growth factor (beta-urogastrone)	-2.41	0.0008

MEF2C	myocyte enhancer factor 2C	-2.12	0.0163
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	-2.06	0.0139
PTPRR	protein tyrosine phosphatase, receptor type, R	-7.61	0.0177
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	-2.02	0.0001
SOS2	son of sevenless homolog 2 (Drosophila)	-2.39	0.0005
Muscle-Down / Alzheimer's disease			
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	-2.10	0.0012
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-2.36	0.0096
Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	-2.04	0.0009
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	-2.34	0.0005
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	-2.06	0.0057
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	-2.06	0.0139

4.3.3.2 Discussion

Cardiac Muscle Contraction and Cardiac Myopathy Pathway

Expression of two L-type voltage-gated calcium channel (VGCC) subunits genes are downregulated in the "cardiac muscle contraction and cardiac myopathy" pathway; CACNA2D1 and CACNA2D3. VGCCs are multi-subunit membrane proteins that regulate calcium influx into excitable cells (i.e. cardiac, smooth, and skeletal muscle cells) [146]. Specific subunits are expressed differentially in different tissue types. CACNA2D1 and CACNA2D3 are α2δ genes that form two linked subunits; α2 and δ (which are both the product of the same gene) that interacts the most with the α1 subunit. Co-expression of the α2δ enhances the level of expression of the α1 subunit and causes an increase in current amplitude, faster activation and inactivation kinetics and a hyperpolarizing shift in the voltage dependence of inactivation.

Other downregulated genes in the same pathway are the ATP2A2 and TPM2.These two genes previously reported as a short-term response gene in muscle in response

to atrophy induction [147]. ATP2A2 encodes one of the SERCA Ca^{2+}-ATPases, which are intracellular pumps located in the sarcoplasmic reticulum of muscle cells which function is coupled with the calcium transient from the cytosol to the sarcoplasmic reticulum lumen, and is involved in calcium sequestration associated with muscular excitation and contraction. TPM2 is a skeletal muscle contractile gene that is reported to cause muscle weakness when mutated [148, 149]. PRKAB2, on the other hand, is the regulatory non-catalytic beta subunit gene for the AMP-activated protein kinase (AMPK). PRKAB2 is a positive regulator of the AMPK that is highly expressed in skeletal muscle [150]. AMPK is an important energy-sensing enzyme that monitors cellular energy status in response to cellular metabolic stresses. PRKAB2 is also reported to be downregulated in a mouse model of severe cachexia [151]. These two genes (TPM2 and PRKAB2) might contribute to the muscular fatigability and decreased mobility that are evident signs of cachexia.

The SGCD gene is one of four known components of the sarcoglycan complex, which is a family of transmembrane proteins and a subcomplex of the dystrophin-glycoprotein complex (DGC). DGC is responsible for connecting the muscle fibre cytoskeleton to the extracellular matrix, preventing muscular damage. Mutations in this gene have been associated with autosomal recessive limb-girdle muscular dystrophy which is characterized by progressive muscle wasting [152].

Titin (TTN) is the third most abundant sarcomeric protein in muscle; an adult human, 80 kg weight, have approximately 0.5 kg of titin [153]. Mutation in TTN gene is known to cause tibial muscular dystrophy which is characterized by weakness and atrophy that is usually confined to the *tibialis* anterior muscle [154]. One of the main functions of titin is to support the contractile elements and maintain muscular

elasticity [154]. Titin also plays a key role in multiple signaling pathways, being a binding site for multiple ligands for a large number of other muscle proteins [154].

The integrin beta 6 (ITGB6) gene is highly expressed in skeletal muscle [155]. It is downregulated in mouse models of cachexia [156]. Integrins are a group of transmemebrane receptors that have two different chains, the alpha and beta subunits that form obligate heterodimers. In mammals, there are eighteen α and eight β subunits [157]. This downregulation of ITGB6 is of unique interest in the context of cachexia because our data shows upregulation in almost every single subunit in all integrins (though not statistically significant in any) except for the ITGB6.

CTNNA3 is one of three alpha-catenin genes [158]. Alpha-T-catenin belongs to the family of cell–cell adhesion molecules and is believed to act as a link between cadherins and actin-containing filaments of the cytoskeleton resulting in strong cell–cell adhesion [158]. It is expressed in cardiomyocytes, skeletal muscle, testis and brain [158]. In silico analysis revealed that the alpha-T-catenin promoter contains several binding sites for cardiac and muscle-specific transcription factors [158]. The two known promoters for CTNNA3 are either unexpressed (GATA4) or significantly downregulated (MEF2C) in our cachectic muscle samples (data from original file) [158].

Glycogen Metabolism Pathway

Another downregulated pathway is the insulin signaling pathway which we rather call "Glycogen metabolism Pathway" due to the function of the genes involved. Three phosphorylase kinase genes (PHKA1, PHKB, and PHKG1) and three protein phosphatase 1 (PP1) genes (PPP1R3A, PPP1R3B, and PPP1R3C) are downregulated. Phosphorylase kinases are a serine/threonine-specific protein

kinase which activates glycogen phosphorylase to release glucose-1-phosphate from glycogen [159]. Protein phosphatases 1, on the other hand, are a class of protein serine/threonine phosphatases that inactivate the active (phosphorylated) form of phosphorylase and also activate the inactive form of glycogen synthase [160]. These two groups of proteins play a crucial role in the regulation of glycogen metabolism. In muscle cells, to power activity instantly when energy is in demand, glycogenolysis is needed to hydrolyze the stores of glycogen to glucose by the sequential removal of glucose monomers. This provided a prompt energy supply for immediate activity in muscle cells [159]. Phosphorylase kinase stimulates glycogen hydrolysis into free glucose by phosphorylating glycogen phosphorylase and stabilizing its active conformation[159]. This is the rate-limiting step in glycogenolysis [159]. Phosphorylase "a" in its active R state has PP1 bound tightly preventing any phosphatase activity of PP1 and maintains the active phosphorylated configuration resulting in a cascade of events that generate the required energy. These changes in gene expression are likely to be involved in the known reduction in glycogen turnover in patients with cancer cachexia, which in turn leads to muscle weakness and rapid fatigue [161]. PRKAB2is a 5'-AMP-activated protein kinase subunit beta-2 that encodes for the regulatory subunit of the AMP-activated protein kinase (AMPK). AMPK is an important energy-sensing enzyme that monitors cellular energy status. This subunit may be a positive regulator of AMPK activity that is highly expressed in skeletal muscle [162]. PRKAB2 knockout mice had reduced maximal and endurance exercise capacity during treadmill running which was also associated with also reduced levels of muscle and liver glycogen [162].

Calcium Signaling Pathway and Long Term Potentiation

CAMK2G encodes for calcium/calmodulin-dependent protein kinase type II gamma, which is involved in sarcoplsamic reticulum Ca^{2+} transport in skeletal muscle [163]. In slow-twitch muscles, is involved in regulation of sarcoplasmic reticulum Ca^{2+} transport and in fast-twitch muscle participates in the control of Ca^{2+} release from the sarcoplasmic reticulum through phosphorylation of the ryanodine receptor-coupling factor triadin. In neurons, may participate in the promotion of dendritic spine and synapse formation and maintenance of synaptic plasticity which enables long-term potentiation (LTP).

PLCB1 encodes for phospholipase C, beta 1 which catalyzes the formation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphatephosphatidylinositol 4,5-bisphosphate. This is an important part of the calcium signaling second messenger system that is crucial for muscle cell growth and differentiation [164].

Parkinson's Disease and Alzheimer's Disease

Three genes are downregulated in Parkinson's and Alzheimer's diseases; NDUFS1, NDUFB5, and ATP5B. Both NDUFS1 and NDUFB5 encode subunits of proteins in the Complex I. Complex I or NADH: ubiquinone oxidoreductase, is the first enzyme of the mitochondrial electron transport chain that initiate cellular respiration or oxidative phosphorylation in the mitochondria in the inner mitochondrial membrane.ATP5B is a subunit of the ATP synthase. It catalyzes ATP synthesis through using the proton motive force created by the electron transport chain across the inner membrane as a source of energy in the process of oxidative phosphorylation.

It is clear that common genes in the Parkinson's and Alzheimer's diseases might sound at the face value to be irrelevant in the context of cancer cachexia. However, these are genes at the core requirements for cellular respiration, and hence energy generation in muscle cells and can clearly contribute to the poor mobility and fatigability in cachectic patients.

4.4 Analysis of Highly Significantly Altered Genes in Cachectic Muscles

From our experience, data generated via high throughput gene expression technology is more likely to be reproduced via other confirmatory gene expression methods such as quantitative RT-PCR only when it has the highest statistical significance levels. Thus, we decided to data-mine our expression data and isolate selected genes with more than two-fold change, highest P-value, and lowest possible FDR. In muscle, 30 genes showed highly significant change in expression (5 upregulated and 25 downregulated: P<0.0005 - P<0.00001, FDR 0.2).

4.4.1 Highly Significantly Upregulated Genes

4.4.1.1 Results

Table 13 shows the 5 highly significantly upregulated genes in cachectic muscle. (P<0.0005 - P<0.00001, FDR 0.2). They include 2 involved with calcium signaling and 2 with cell matrix interactions, and one transcriptional regulator. Table 13 categorizes individual gene function into respective functional domain. It also shows the full gene name, fold change, and the P-value.

Table 13: Highly Significantly Upregulated Genes in Cachectic Skeletal Muscle.

Gene Symbol	Gene Full Name	Fold change	P-Value
Signaling			
PLCXD1	phosphatidylinositol-specific phospholipase C, X domain containing 1	4.60	<0.00001
PITPNM2	phosphatidylinositol transfer protein, membrane-associated 2	3.82	0.00005
Cell Matrix interaction			
ESAM	endothelial cell adhesion molecule	3.39	0.0004
PXN	Paxillin	2.16	<0.00001
Transcription			
MLLT1	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1	2.36	< 0.0003

4.4.1.2 Discussion

Calcium Signaling Genes

Two genes which are suggested to be involved in signaling are significantly upregulated in cachectic muscles; PLCXD1 and PITPNM2. PLCXD1 codes for phosphatidylinositol-specific phospholipase C, X domain containing 1. Phospholipase C (PLC) is a group of enzymes with differences in domain structure, that cleave the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains bound to the membrane, and IP_3 is released as a soluble structure into the cytosol to bind to IP_3 receptors, particularly calcium channels in the smooth endoplasmic reticulum. This causes the cytosolic concentration of calcium to increase, causing a cascade of intracellular changes and activity [165, 166]. In turn, calcium and DAG together work to activate protein kinase C, which goes on to phosphorylate other molecules, leading to altered cellular activity [165]. Phospholipases are ubiquitously expressed and have diverse biological functions [166]. In tumor cells, PLCXD1 suppress

growth when transfected in melanoma cell line [167]. There are no further functional studies characterizing this gene, however, like other phospholipases, it undoubtedly plays an important role in cellular signaling specifically through calcium release, which is important in modulating many muscle-related functions.

The second upregulated gene is PITPNM2 which codes for phosphatidylinositol transfer protein (PITP) membrane-associated 2 protein. PITP proteins are ubiquitous cytosolic domain involved in transport of phospholipids from their site of synthesis in the endoplasmic reticulum and Golgi to other cell membranes. PITPNM2 is not functionally characterized yet. However, PITPα, a member of the PITP family that was also upregulated in our study, was identified as an essential component in ensuring substrate supply to phospholipase C by dictating the rate of inositol trisphosphate production by promoting the synthesis of PIP_2 [168].

Thus, these two genes could be functioning through IP_3/DAG pathway. Thus, PITPNM2 might be promoting PIP_2 synthesis and making it readily available for PLC (in our case is PLCXD1) which in turn cleaves the PIP_2 into PIP_3 and DAG. Both of which work as key second messengers, which via multiple cascades of events, causes Ca^{2+} transient into the cytosol. Calcium, in turn, participates in the cascade by activating proteins.

Cell Matrix Interaction

Two cell matric interaction genes were markedly upregulated in cachectic muscles; ESAM and PXN. ESAM encodes ~~is~~ an endothelial cell-selective adhesion molecule. Though ESAM has never been studied, *per se*, in skeletal muscle, it has been studied in the context of coronary vascular disease (CVD) and kidney disease [169]. Skeletal muscle vasculature is of considerable importance. Around 20% of cardiac output is directed toward skeletal muscle vasculature during rest and more than

80% of cardiac output can be directed to contracting muscles. Increased expression of ESAM may indicate early endothelial dysfunction that precedes kidney function decline among individuals with established CVD [169]. Also, induced endothelial dysfunction in skeletal muscle arterioles compromise glucose utilization and facilitates the development of hypertension in diabetes [170]. Muscular vasculature is of a great importance to muscle development, maintenance, and function. The dysfunctional vascular endothelium associated with higher ESAM in CVD and kidney disease may indicate the importance of ESAM in healthy muscular vasculature. Thus, higher ESAM in the muscle of cachectic patients may have a deleterious effect in vasculature compromising blood flow and/or glucose utilization.

Paxillin (encoded by PXN) is a signal transduction, adaptor, cytoskeletal, extracellular matrix (focal adhesion) protein involved in actin-membrane attachment [171]. The C-terminal region of paxillin contains four LIM domains that target paxillin to focal adhesions. It is presumed through a direct association with the cytoplasmic tail of beta-integrin [171]. The N-terminal region of paxillin is rich in protein–protein interaction sites [171].

One might wonder why two cell matrix interaction proteins are upregulated in muscles of patients with early cachexia. However, muscle hypertrophy (not hyperplasia) in both cardiac and skeletal muscle is an adaptation to regular, increasing workloads that exceed the preexisting capacity of the muscle fibers. Thus hypertrophy allows cardiac muscle to pump blood more effectively, while skeletal muscle becomes more efficient at transmitting forces through its tendonous attachments to bones [172]. Paxillin has been associated with the regulation of cytoskeletal organization, focal adhesion formation, cell migration and cell motility [173]. Paxillin is also localized to the myotendinous and neuromuscular junctions of

skeletal muscle [174]. While expression of non-phosphorylatable paxillin mutants in canine tracheal smooth muscle inhibits tension development [173].

Wasted patients are usually weaker. That said, normal/regular activities can be envisioned as progressive overload causing intermittent levels of stress to skeletal muscle. Thus, as an adaptation to wasting, paxillin may be attempting but failing to promote hypertrophy in this situation. Muscular hypertrophy means its ability to adapt by increasing the size and amount of contractile proteins (cell matrix interaction proteins), which comprise the myofibrils within each muscle fiber, leading to an increase in the size of the individual muscle fibers and their consequent force production [172]. That is similar to previously reported evidence of the increased in paxillin expression along other proteins upon the increased mechanical load per unit of cross-sectional area during short period of an increasing load [175].

Transcriptional Regulation

Transcriptional regulation is a complicated series of mechanisms used by cells to increase or decrease the expression of specific gene products. These sophisticated programs of gene expression serve several purposes of which developmental and adaptive responses are just a few. Transcriptional regulation is a versatile and adaptable mechanism allowing the cell to express proteins when needed. For these reasons, upregulation of genes involved in transcriptional regulation is expected in the context of wasting muscle. Whether cachectic muscles die via apoptosis or necrosis, attempt to regenerate or differentiate, or just change in its morphogenic features, regulation of transcription is definitely needed.

MLLT1 gene, or myeloid/lymphoid or mixed-lineage leukemia translocated to 1, codes for a transcriptional regulator that is highly significantly upregulated in cachectic muscle. MLLT1 has never been studied in the context of cachexia nor

muscular transcriptional regulation. However, evidence suggests an important developmental role of this gene. MLLT1 has nuclear localization signal, and expressed widely in variant tissues suggesting its role as a coactivator activating individual lineage developmental programs [176]. MLLT1 has been shown to be required for early embryonic development in the mouse, as knock-out embryos are non-viable [176]. Also, an enhanced MLLT1 expression plays a role in testicular germ cell tumors [177]. Thus, MLLT1 gene could be an activator for gene expression of multiple genes that can either explain the pathology of wasting and/or illustrate an early compensatory role to resist wasting. Identifying which of these roles MLLT1 plays would require further investigation.

4.4.2 Highly Significantly Downregulated Genes

4.4.2.1 Results

Table 14 shows the 5 highly significantly downregulated genes in cachectic muscle. ($P<0.0005$ - $P<0.00001$, FDR 0.2). Analysis of the 25 downregulated genes involved include 7 that are involved with metabolism (5 of which are mitochondrial); 4 with signaling; 4 with ubiquitination; and 3 with intracellular trafficking. There was marked downregulation of multiple genes involved in glycogen metabolism which correlates with the lack of glycogen, muscle weakness, and fatigue; characteristic of cachexia. Table 14, also categorizes individual gene function into respective functional domain. It also shows the full gene name, fold change, and the P-value.

Table 14: Highly Significantly Downregulated Genes in Cachectic Skeletal Muscle.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Mitochondrial Metabolism			
SUCLA2	succinate-CoA ligase, ADP-forming, beta subunit	-2.33	<0.0003
ALDH5A1	aldehyde dehydrogenase 5 family, member A1	-4.16	<0.00001
ACADM	acyl-CoA dehydrogenase, C-4 to C-12 straight chain	-3.64	<0.0004
NARS2	asparaginyl-tRNAsynthetase 2, mitochondrial (putative)	-2.05	0.0003
NNT	nicotinamide nucleotide transhydrogenase	-2.62	0.00003
Metabolism			
PRKAB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	-2.64	0.0001
AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	-3.51	0.0002
Signaling			
PLCL1	phospholipase C-like 1	-3.64	<0.00008
NXPE3	neurexophilin and PC-esterase domain family, member 3	-2.90	<0.00005
ZFYVE9	zinc finger, FYVE domain containing 9	-2.07	0.0002
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	-2.02	<0.00008
ZNF41	zinc finger protein 41	-2.12	0.0003
Muscle Contraction			
PPP1R12B	protein phosphatase 1, regulatory subunit 12B	-2.10	0.00005
Cell Matrix Interaction			
ITGB6	integrin, beta 6	-5.09	0.0002
Ubiquitination			
WWP1	WW domain containing E3 ubiquitin protein ligase 1	-3.37	0.00001
ZYG11B	zyg-11 family member B, cell cycle regulator	-3.01	0.00007
FBXL17	F-box and leucine-rich repeat protein 17	-2.38	0.00005
DCAF6	DDB1 and CUL4 associated factor 6	-2.59	0.0004
Intacellular Trafficking			
RAB9B	RAB9B, member RAS oncogene family	-3.10	<0.00009
RAB10	RAB10, member RAS oncogene family	-2.18	0.00006
CLASP2	cytoplasmic linker associated protein 2	-2.30	0.0004
Apolipoproteins			
VLDLR	very low density lipoprotein receptor	-2.9	<0.00005
APOOL	apolipoprotein O-like	-2.06	<0.0003
Uncharachterized			
C1orf192	chromosome 1 open reading frame 192	-3.87	<0.0004

4.4.2.2 Discussion

Mitochondrial Metabolism

SUCLA2 (succinyl-CoA ligase [ADP-forming] mitochondrial) encodes for ADP-forming succinyl-CoA synthetase, a mitochondrial matrix enzyme which catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA [178]. It plays a key role as one of the catalysts involved in the citric acid cycle, a central pathway in cellular metabolism [165]. Decreased SUCLA2 activity due to mutation was found in muscle mitochondria of patients with encephalomyopathy and mtDNA depletion [179].Myopathy is a disease of dysfunctional muscle fibers resulting in muscular weakness.

ALDH5A1 codes for succinate-semialdehyde dehydrogenase, mitochondrial, an enzyme that belongs to the aldehyde dehydrogenase family of proteins [180]. ALDH5A1 deficiency results in muscle hypotonia and developmental delay [181, 182]. Hypotonia is a state of low muscle tone (the amount of tension or resistance to stretch in a muscle), often involving reduced muscle strength. Mice with a homozygous knockout of ALDH5A1 exhibit symptoms including reduced body weight [183].

ACADM encodes for an acyl-coenzyme a dehydrogenase that is important for hydrolyzing medium-chain fatty acids to produce energy, especially during periods of fasting [184]. Fatty acid oxidation spares glucose during fasting and is also required for amino acid metabolism, which is essential for the maintenance of adequate glucose production [185]. With a deficiency of this enzyme, medium-chain fatty acids cannot be hydrolysed then these fatty acids are not converted into energy, leading to weakness, lethargy, and low blood sugar [186]. Training increases fatty acid oxidation and skeletal muscle oxidative capacity by increasing

expression of ACADM which results in increased use fatty acids to enhance skeletal muscle oxidative capacity [187].

NARS2 codes for asparaginyl-tRNA synthetase 2 [188]. This class-II synthetase is specific for alanine, asparagine, aspartic acid, glycine, histidine, lysine, phenylalanine, proline, serine, and threonine [189]. NARS2 mutations cause severe myopathy characterized by proximal muscle weakness, severe amyotrophy (progressive wasting of muscle tissues), excessive fatigability, dysarthria, paresis of facial muscles, and ptosis [190]. Skeletal muscle biopsies show atrophic fibers, ragged-red fibers, and enlarged mitochondria [190]. Other NARS2 mutations cause Leigh Syndrome, which results in hypotonia (low muscle tone and strength), dystonia (involuntary, sustained muscle contraction), and ataxia (lack of control over movement). Mutations in mitochondrial seryl-tRNAsynthetase, another member of the class II amino-acyl tRNA family, cause severe cachexia [191].

NNT codes for nicotinamide nucleotide transhydrogenase, an integral protein of the inner mitochondrial membrane. Under physiological conditions, this enzyme uses energy from the mitochondrial proton gradient to produce high concentrations of NADPH. The resulting NADPH is used for biosynthesis and in free radical detoxification [192].

Metabolism

As mentioned above PRKAB2 is the regulatory non-catalytic beta subunit gene for the AMP-activated protein kinase (AMPK). PRKAB2 is a positive regulator of the AMPK that is highly expressed in skeletal muscle [150]. AMPK is an important energy-sensing enzyme that monitors cellular energy status. PRKAB2 is also reported to be downregulated in a mouse model of severe cachexia [151]. PRKAB2 knockout mice had reduced maximal and endurance exercise capacity during

treadmill running which was also associated with also reduced levels of muscle and liver glycogen [162]. Thus, PRKAB2 might contribute to the muscular fatigability and the decreased mobility that are evident signs of cachexia.

AGL codes for the glycogen debranching enzyme which facilitates the hydrolysis of glycogen in through glucosyltransferase and glucosidase activity [193]. Mutation in AGL causes one of the glycogen storage diseases (type III) and results in peripheral muscular impairment which can vary from minimal to severe [194] as well as muscle weakness [195].

Signaling

PLCL1 codes for phospholipase C-Like 1, one of several phosphoinositide phospholipase C enzymes that play an important role in signal transduction [196]. These enzymes participate in phosphatidylinositol 4,5-bisphosphate (PIP_2) metabolism in a calcium-dependent manner. All members in this family are capable of catalyzing the hydrolysis of PIP_2 into two important second messenger molecules (DAG and IP_3), which go on to alter multiple cellular responses such as proliferation, differentiation, apoptosis, cytoskeleton remodeling, vesicular trafficking, ion channel conductance, endocrine function and neurotransmission.

However, it is not the only member in the PLC family that is downregulated. PLCB1 is also downregulated in multiple muscle downregulated KEGG pathways (Table 12). This is important since calcium signaling is important for muscle contraction and thus, it affects muscle contraction and eventually causes weakness. NXPE3 codes for neurexophilin and PC-esterase domain family, member 3 which is another uncharacterized gene [197].

ZFYVE9 encodes a zinc finger FYVE domain-containing protein 9 or SARA (SMAD anchor for receptor activation) [198]. SARA contains a double zinc finger (FYVE domain). SARA is an anchoring protein involved in transforming growth factor beta (TGF-β) signaling. It binds to the R-SMADs SMAD2 and SMAD3 [199].It facilitates the phosphorylation of the R-SMAD, which subsequently dissociates form SARA and the receptor and binds a co-SMAD where they enter the nucleus as transcription factors. All SMADs are downstream to myostatin (and TGF-β and all of its related proteins), which is believed to be activated in wasting muscle. However, our data shows significantly downregulatedmyostatin in cachectic muscles (as will be explained in details later). Thus, a downregulated ZFYVE9 may be further evidence of an adaptive response to myostatin signaling in cachexia.

RPS6KA3 codes for the ribosomal protein S6 kinase which is a member of the RSK (ribosomal S6 kinase) family of serine/threonine kinases. This kinase phosphorylates various substrates, including members of the mitogen-activated kinase (MAPK) signaling pathway (which one of the downregulated KEGG pathways in cachectic muscles as above) [200]. The activity of this enzyme has been implicated in controlling cell growth and differentiation. In muscle cells, it mediates cellular differentiation through regulation of NFAT3 [201]. Mutations in this gene are associated with Coffin-Lowry syndrome [202], which is characterized by growth and psychomotor retardation, hypotonia, and progressive skeletal changes [203]. Also, RPS6KA3 knockout mice have altered extracellular signal-regulated kinase signaling and glycogen metabolism in skeletal muscle, 10% reduction in weight, and are 14% shorter than wild-type littermates [204]. A study of patients with Coffin-Lowry syndrome showed episodes of sudden, non-epileptic collapse with atonia, described as a form of cataplexy (Cataplexy comprises an abrupt, complete, or

partial loss of voluntary muscle tone and is usually seen in response to strong emotion) [205].

ZNF41 encodes for zinc finger protein 41, a member of zinc finger family of transcription factors [206]. So far, little is known about ZNF41. However, having one of the highest expressions of ZNF41 in skeletal muscle [207] is an indicator of its transcriptional importance.

Muscle Contraction

PPP1R3A codes for the regulatory subunit and myosin binding subunits of myosin light chain phosphatase (MLCP), which is abundantly expressed in skeletal and cardiac muscle [208]. This gene plays an important regulatory role in muscle contraction.

Ubiquitination

Ubiquitination (also known as ubiquitylation) is an enzymatic, post-translational modification (PTM) process in which ubiquitin protein is attached to a substrate protein, targeting the protein for degradation. This results in the addition of one (monoubiquitination) or multiple (polyubiquitination) ubiquitin molecules to the substrate protein [209]. The ubiquitination system functions in a wide variety of cellular processes.

WWP1 codes for a NEDD4-like E3 ubiquitin-protein ligase, which belongs to a family of E3 ubiquitin-ligases that regulate key trafficking decisions, including targeting of proteins to proteosomes or lysosomes. Interestingly, studies aimed at understanding chicken muscular dystrophy showed mutations in WWP1 in dystrophic chicken [210]. Another study on chickens treated with dexamethasone (which causes wasting) found that WWP1 expression was dependent on the duration of

dexamethasone treatment; short-term resulted in decreased WWP1 expression, while long-term increased WWP1 expression [211]. Detection of highly significant downregulation of WWP1 in cachectic muscles in our patients suggests an adaptive response to the early cachexia.

ZYG11B codes for zyg-11 family member B, a cell cycle regulator protein. It probably acts as target recruitment subunit in the E3 ubiquitin ligase complex ZYG11B-CUL2-Elongin BC [212]. One study showed that erythropoietin (EPO) functional knock-out mice have lower ZYG11B expression in skeletal muscle compared to controls [213]. The EPO receptor is highly expressed in muscle and may suggest important role of EPO in stimulating skeletal muscle regeneration [213].

FBXL17 codes for the F-box and leucine-rich repeat protein 17, a member of the F-box protein family. Complexes, formed by the presence of F-box proteins act as protein-ubiquitin ligases [214]. There is no further information regarding this gene.

Intacellular Trafficking

RAB9B codes for a member of sub-family of the RAS small guanosine triphosphate (GTP)-binding proteins that regulate membrane trafficking. The encoded protein may be involved in endosome-to-Golgi transport [215]. Diseases associated with RAB9B include pelizaeus-merzbacher disease which has early onset hypotonia. With progressive disease, even more severe muscular involvement is evident.

RAB10 codes for another member in the RAS oncogene family [216]. RAB10 has the highest expression in heart and skeletal muscle [217]. It is involved in the insulin signaling cascade that causes intracellular vesicles containing glucose transporter-4

(GLUT4) to translocate to and fuse with the plasma membrane and enhance glucose uptake [218, 219].

CLASP2 codes for cytoplasmic linker associated protein 2, a microtubule plus-end tracking protein that promotes the stabilization of dynamic microtubules. It is also required for the polarization of the cytoplasmic microtubule arrays in migrating cells towards the leading edge of the cell. Like RAB10, CLASP2 has a role in insulin signaling and glucose uptake in myocytes and adipocytes [220]. CLASP2 colocalizes with the glucose transporter GLUT4 at the plasma membrane within areas of insulin-mediated cortical actin remodeling [220]. CLASP2 knockdown inhibits insulin-stimulated GLUT4 translocation and glucose transport [220].

This downregulation of this pathway undoubtedly contributes to the insulin resistance and muscle fatigue in cachectic patients.

On the other hand, a hallmark of the neuromuscular junction (NMJ) is the high density of acetylcholine receptors (AChRs) in the postsynaptic muscle membrane [221, 222]. CLASP2 mediates microtubule capturing at the synaptic membrane for the maintenance of a normal neuromuscular phenotype [222, 223]. The absence of CLASP2 impairs the maintenance of the neuromuscular junction with a decreased subsynaptic membrane in muscles, and a decrease of synaptic AChRs [224]. The significant downregulation of CLASP2 in cachectic muscles will alter the vitality of the NMJ and it turn cause impairment of muscle excitability [224]. Even more interestingly, the regulation of CLASP2 strictly controlled by agrin (AGRN) [223], which is itself downregulated in the KEGG pathways as discussed above.

Apolipoproteins

VLDLR is a very-low-density-lipoprotein receptor; a transmembrane lipoprotein receptor of the low-density-lipoprotein (LDL) receptor family. VLDLR is widely and highly expressed in heart, muscle, and adipose tissues, which are active in fatty acid metabolism [225], so the receptor targets lipoproteins to these tissues [226]. Although the mechanism of VLDLR function in muscle is not fully understood, the defective clearance of triglyceride-rich lipoproteins leads to impaired energy metabolism, reduced exercise capacity, cachexia, and malnutrition syndrome in patients with advanced chronic kidney disease [227]. Also, reduced levels of lipoprotein lipase and VLDLR contribute to cachexia and reduced exercise capacity [227]. Mutations of this gene causes type I lissencephaly; a condition of which one of the symptoms is hypotonia [228].

APOOL encodes an apolipoprotein O-like protein. The apoliprotein 0-like domain is found in the protein component of circulating lipoprotein complexes. Apolipoprotein O is a cardiolipin-binding component of the Mitofilin/MINOS protein complex determining cristae morphology in mitochondria (the folds in the inner membrane of a mitochondrion) [229]. Downregulation of APOOL impairs mitochondrial respiration and causes major alterations in cristae morphology; which are only altered under pathological conditions [229]. The important paralog; APOO [229]. APOO transgenic mouse fed a high-fat diet exhibited depressed ventricular function with reduced fractional shortening and ejection fraction, and the myocardial sections revealed mitochondrial degenerative changes [230]. Hence, APOO represents a link between impaired mitochondrial function and cardiomyopathy onset causing impaired contractility [230]. Furthermore, APOO enhances mitochondrial uncoupling and respiration, both of which were reduced by targeted knockdown of APOO [230]. If

that is the case in cardiac muscle, then APOOL downregulation in our cachectic muscle could be an early sign of impairment in skeletal muscle contractility as a result of damaged mitochondria.

4.5 Anticipated Results from Previous Studies

Preclinical studies have a few produced findings in multiple settings that attempt to explain the pathogenesis of cancer cachexia. Without exception, these findings so far have failed to explain cachexia in the clinical setting. This section aims to look at some of the prominent findings from clinical and preclinical models of cancer cachexia in order to see its reproducibility in our sequencing data.

4.5.1 Preclinical Findings

4.5.1.1 Atrophy-Specific Ubiquitin Ligases

It is long believed that cachexia is a result of a negative nitrogen balance caused by decreased protein synthesis combined with increased protein degradation. The ubiquitin proteasome pathway is the main protein degradation pathway involved. Two genes were repeatedly found to be downregulated in preclinical models of cachexia; FBXO32 (atrogin-1/MAFbx) and TRIM63 (MURF1).

FBXO32 codes for F-box only protein 32 [231], while TRIM63 codes for E3 ubiquitin-protein ligase also known as MURF1. These are two atrophy-specific ubiquitin ligases that are two of the several key regulators of the ubiquitin proteasome degradation pathway. Both of the genes mediate sarcomeric breakdown and inhibit protein synthesis [232]. FBXO32 expression was significantly upregulated in atrophying muscles in cachectic rats and mice secondary to fasting, diabetes, renal failure or experimental cancer [231]. This upregulation was also confirmed *in vitro* in a murine skeletal muscle cell line [233]. Both MuRF1 and MAFbx were substantially

upregulated in rat skeletal muscle after immobilization, denervation, hind limb suspension or treatment with interleukin-1 or dexamethasone; conditions that are all associated with a significant loss of muscle mass [234].

In our data of cachectic muscle, nether of the two genes were found to be upregulated. Indeed, in contrast, FBXO32 was even found to be significantly downregulated. Similarly, the two major microarray experiments documenting gene expression changes in human muscle biopsies of cachectic patients also found no change in the expression level of either of these two genes, which were long believed to be key players in the pathogenesis of cachexia [235, 236]. The downregulation of FBXO32 was not a surprise since the downregulation of the ubiquitin pathway was also evident with 4 other highly significantly downregulated genes (as discussed earlier). Downregulation of this protein degradation pathway may be a compensatory mechanism to defend against wasting in the cachectic subjects.

Myostatin (MSTN) and its associated receptor activin (ACVR2B) have recently stolen the limelight as a possible cause of cachexia. MSTN or growth and differentiation factor 8 (GDF8) is a TGF-β family member. Mutations in the MSTN gene causes muscular hypertrophy in animals [237] as well as humans [238]. On the other hand, overexpression of MSTN caused pronounced muscular atrophy [239].

MSTN which is mainly synthesized in skeletal muscle cells works by activating ACVR2B receptor that in turn, recruits an Alk family kinase resulting in activation of a SMAD2 and SMAD3 transcription factor complex and leading to activation of muscle loss signals that are as yet not fully understood [240]. For that, many attempts have been tried to block this signals in atrophying muscle. For example,

blocking the ACVR2B receptor in mice can reverse cachexia and muscle wasting [241].

Neither the present study nor the previous clinical expression studies have shown any upregulation in MSTN, its receptor, or any downstream elements. In contrast, our data shows significant downregulation in both, MSTN and ACVR2B, which again may reflect the bodies' complicated response to try to prevent wasting. Based on the previous animal studies, several clinical trials are currently investigating the outcome of blocking MSTN with monoclonal antibodies or inhibiting elements of its downstream signaling. We believe that because the receptor and downstream elements are downregulated that these trials are desined to failure. However, it might give some reversal to the syndrome in unique settings. It should be noted that some cancers appear themselves to produce MSTN [242]. If this is found to be a general phenomenon then downregulation of muscle MSTN and its receptor may be considered adaptive responses to protect the muscle from tumor-derived MSTN.

A very interesting finding from our data is the significant overexpression of GDF15. GDF15 is a growth and development factor 15 (NAG-1; MIC-1). It belongs to the transforming growth factor beta superfamily [243] that has a role in regulating inflammatory and apoptotic pathways in injured tissues and during disease processes [244]. In advanced cancer, high serum levels of GDF15 can lead to cachexia via central regulation of appetite [245]. In mice, cachexia induced by GDF15 overexpression can be reversed by anti-GDF15 monoclonal antibodies [245]. Our data shows a very interesting significantly upregulated GDF15 in cachectic muscle (12.9 fold change; P-value 0.028). This level of change is reported for the first time within the muscle, indicating its probable role in cachexia pathogenesis that warrants further investigation.

4.5.2 Previous Clinical Findings

4.5.2.1 Inflammatory Cytokines and Tumor Specific Factors

The proteolysis-inducing factor (PIF) was isolated from urine using monoclonal antibody in murine cachexia model [246] as well as human subjects with pancreatic cancer [247]. PIF is believed to possess an ability to degrade skeletal muscle. Although PIF has been linked with weight loss in patients, other groups have failed in reproducing the above finding; hence PIF was not redetected [248]. Despite our knowledge that PIF was originally captured in subjects' urine, we were still interested in checking its expression within the cachectic muscles. PIF (DCD; dermicidin) was not detected in our sequencing data. It should be noted that if the tumor itself is the source of PIF, we would not have expected to detect it in the muscle tissue; so we cannot discount its existence at this time.

Many inflammatory cytokines have long been implicated in muscle wasting. Those include tumor necrosis factor-alpha (TNF), interleukin-6 (IL6), and interferon-gamma (IFNG). Preclinical studies showed significant contribution of each of these cytokines in the development of cachexia [96, 110, 111]. Unlike TNF and IFNG which were not found to correlate to weight loss in human [112], circulating levels of IL-6 have been shown to correlate with weight loss in cancer patients [249]. In the present study, the expression levels of these three cytokines in cachectic muscle were not statistically significant (Table 15). However, for IL6 and IFNG expression was higher, but not significant. It is possible that expression of these cytokines is increased in some but not all cachectic patients.

Table 15: Inflammatory Cytokines and Tumor Specific Factors.

Gene Full Name	Gene	Fold Change	P-Value
Tumor necrosis factors-alpha	TNF	1.12	0.8997
Interleukin-6	IL6	6.96	0.2241
Interferon-gamma	IFNG	5.47	0.1819
Dermcidin/proteolysis-inducing factor	DCD/PIF	Not expressed	

4.5.3 Results from Previous Genome Wide Expression Analysis

Stephens et al. (2010) were the first group to look at genome wide expression profiling in muscle biopsies obtained from weight losing cancer patients compared to weight stable non-cancer controls [235]. Using muscle biopsies from the rectus abdominis, eight genes were found to be differentially expressed by the microarray that were also confirmed by RT-PCR. Changes in expression of these eight genes were then rechecked in two different groups of muscles (diaphragm and *vastus lateralis*) from a different center. Only two genes were reproducible from the 8 genes; TIE1 and CAMK2B. Both genes are "endurance exercise"-activated genes. Overexpression of such two genes may signal muscles attempt to maintain its viability through activation of pathways usually active during moderate endurance activity [235]. We looked at the expression level of all the genes that was found to be significant in the previous microarray study 1 (Table 16). Only TIE1 was found to be significantly upregulated in the present study, in-line with what was previously reported. How HTIE1 functions is still unknown, however it is an exercise activated gene, usually upregulated in response to prolonged training [250].

Table 16: Results of Significantly Altered Genes in Previous Microarray Study 1.

Gene Full Name	Gene	Fold Change	P-Value
Adenomatosis polyposis coli down-regulated 1	APCDD1	3.50	0.1063
Calcium/calmodulin-dependent protein kinase II beta	CAMK2B	1.00	0.9963
Eukaryotic translation initiation factor 3, subunit I	EIF3I	-1.11	0.5461
Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	2.67	0.0936
NudC nuclear distribution protein	NUDC	-1.16	0.5105
Polymerase (RNA) mitochondrial (DNA directed)	POLRMT	1.25	0.7541
Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	**TIE1**	**4.50**	**0.0218**
Tuberous sclerosis 2	TSC2	1.81	0.031
Serum/glucocorticoid regulated kinase 1	SGK1	4.52	0.3278

In 2012, the same group of investigators of the above study reattempted another microarray-based, genome-wide expression analysis of quadriceps muscle biopsies in single biopsies from healthy controls and in paired biopsies (pre-resection baseline and 8 month post-resection follow-up) from cachectic cancer patients [236]. They detected 10 genes where expression was significantly changed (Table 17).

Table 17: Results of Significantly Altered Genes in Previous Microarray Study 2.

Gene Full Name	Gene	Fold Change	P-Value
Cartilage oligomeric matrix protein	COMP	Not expressed	
Adiponectin, C1Q and collagen domain containing	ADIPOQ	6.98	0.3605
Matrix metallopeptidase 3	MMP3	Not expressed	
Phosphoenolpyruvatecarboxykinase 1 (soluble)	PCK1	15.29	0.2891
Angiopoietin-like 7	ANGPTL7	1.75	0.3664
Heat shock protein 90kDa alpha (cytosolic), class B member 1	HSP90AB1	-1.29	0.2028
Solute carrier family 25 (mitochondrial iron transporter), member 37	SLC25A37	-1.24	0.229
Prospero homeobox 1	PROX1	-1.4	0.4232
Regulator of calcineurin 1	RCAN1	2.41	0.3536
Histidine triad nucleotide binding protein 3	**HINT3**	**-2.45**	**0.0061**

Of the genes studied, the only significantly changed gene that is common with the present study is the HINT3. HINT3 is a histidine triad nucleotide binding protein 3 [251]. It is an uncharacterized gene. However, it has been recently found to be expressed in skeletal muscle in the chimpanzee [252].

4.6 Verified Results Using Real-Time Reverse Transcription PCR

Despite of the high reliance of the quantitative capability of the RNA sequencing results, it is important to verify part of the expression results using a different method. A cherry-picked selection of genes that are of a high significance and high fold-change were picked. Confirming the difference of high fold-change expression data can allow us to check and verify them later-on at protein level. Four upregulated genes; COL4A2, NRXN2, NOTCH1, and AGRN, and two downregulated genes; NDUFS1, and PTPRR were chosen.

4.6.1 Results

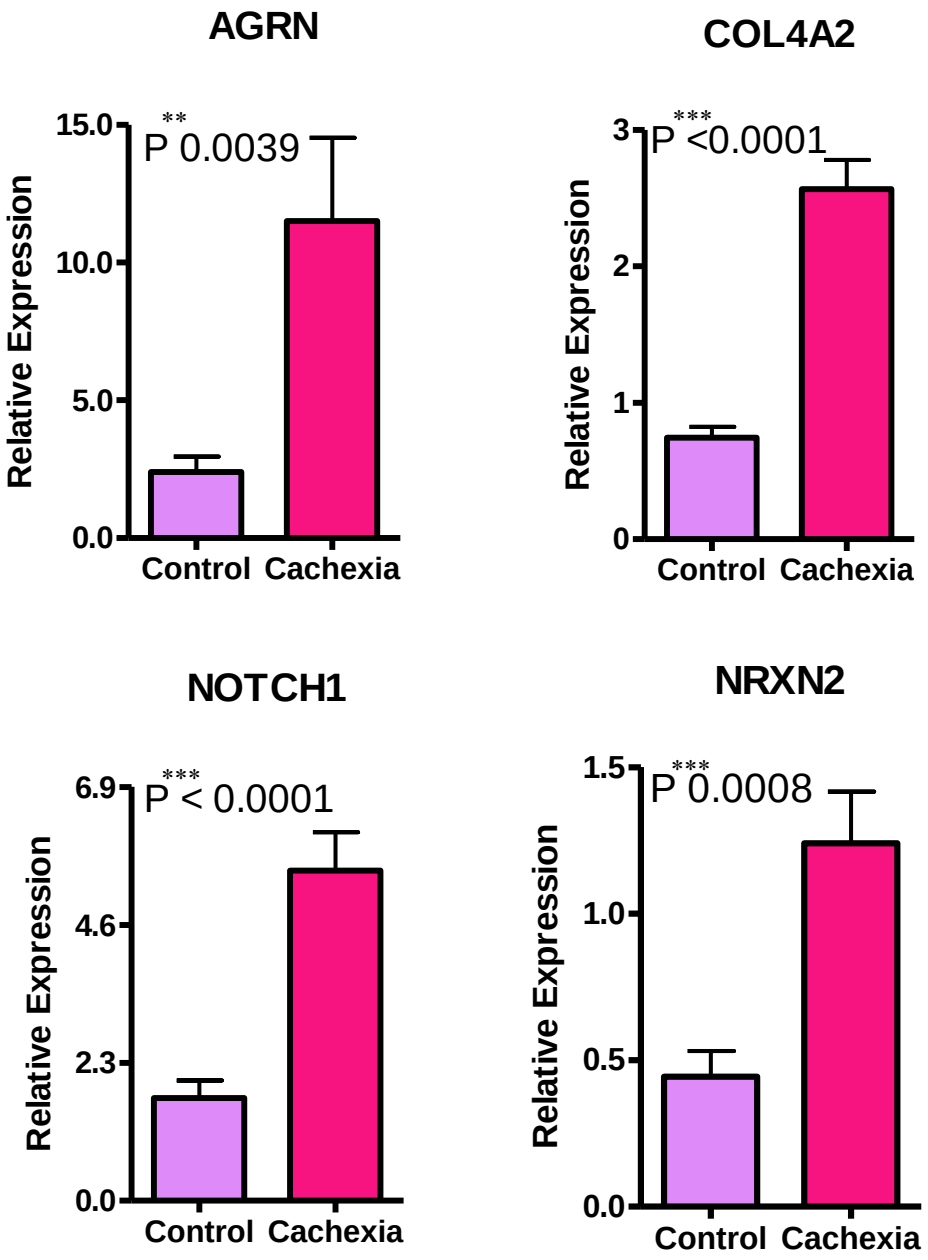

Figure 3: Upregulation of Expression of Selected Genes Confirmed by Real-Time RT-PCR in Cachectic Skeletal Muscle.

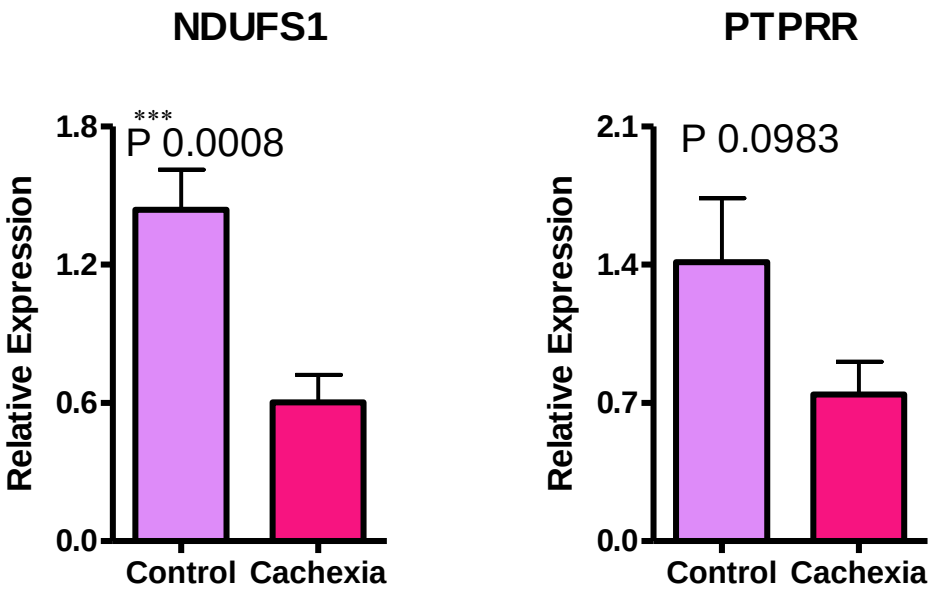

Figure 4: Downregulation of Expression of Selected Genes Confirmed by Real-Time RT-PCR in Cachectic Skeletal Muscle.

All chosen genes that were previously shown to be upregulated/downregulated were reproducible via real-time RT-PCR (Figure 3 and 4).

4.6.2 Discussion

Expressions of four upregulated genes that are statistically significant were confirmed via real-time RT-PCR.

AGRN (Agrin) and COL4A2 (collagen type 4 alpha 2) (section 4.3.2.2) are elements in the extracellular matrix-receptor interaction. AGRN fragments are under investigation as a possible biomarker for sarcopenia [136] and was also found to be upregulated in cachectic muscles. Also, COL4A2 is a component of the myocyte basal lamina [137].

However, the other two genes; NOTCH1 receptor (section 4.3.2.2), an activators of the notch pathway [131, 132], and NRXN2 (a cell adhesion molecules) are two poorly characterized genes in skeletal muscle and their confirmed upregulation warrant further research.

The confirmed downregulated genes were the NDUFS1 and the PTPRR. The NDUFS1 is upregulated in the Parkinson's and Alzheimer's diseases (section 4.3.3.2). NDUFS1 encodes subunit of proteins in the Complex I. Complex I or NADH: ubiquinone oxidoreductase, is the first enzyme of the mitochondrial electron transport chain that initiate cellular respiration or oxidative phosphorylation in the mitochondria in the inner mitochondrial membrane.ATP5B is a subunit of the ATP synthase. It catalyzes ATP synthesis through using the proton motive force created by the electron transport chain across the inner membrane as a source of energy in the process of oxidative phosphorylation.

PTPRR is a protein tyrosine phosphatase, receptor type, R. PTPRR gene is a MAPK signaling pathwaygenethat is uncharacterized in the skeletal muscle and is correlated for the first time to cachexia.

Chapter 5: Results and Discussions – Adipose Tissue

5.1 Demographic and Biochemical Characteristics of Subjects

5.1.1 Results

Demographic and biochemical details of patients for fat cachexia samples (FS) and fat weight-stable controls (FC) are shown in bellow tables. The tables show data for 6 samples in each group. Samples were analyzed based on weight, BMI, some nutritional parameters, inflammatory markers and other related demographics.

Table 18 shows subjects oncological diagnoses for visceral adipose tissue biopsies. All patients were with histologically confirmed malignancies. Age and gender distribution were not significantly different between cachexia fat cases (FS) and weight-stable controls (FC) (Table 19). All biochemical nutritional parameters (i.e. hemoglobin, total protein, albumin, urea, and creatinine) and inflammatory markers (i.e. WBC and CRP) were not significantly different between both groups (Table 19). Baseline weights and BMIs in the cachexia group did not differ significantly from the control. The time range between current (at time of biopsy) and baseline weights (preceding date of biopsy) was 3-6 months. Mean weight loss from baseline in the cachexia group was 4.6 kg which is 6.5% of baseline body weight (i.e. 3-6 months before the date of sample collection). Amount and percentage of weight loss was significantly different from controls that had relatively stable weights; P-value of 0.0001 and < 0.0001, respectively.

Table 18: Subjects' Diagnoses for Adipose Tissue Biopsies.

Cachexia Fat Cases (FS)	**Weight-stable Fat Controls (FC)**
FS1: Malignant Neoplasm of Rectum	FC3: Malignant Neoplasm of Colon
FS2: Malignant Neoplasm of Stomach	FC5: Malignant Neoplasm of Colon
FS3: Malignant Neoplasm of Rectum	FC6: Malignant Neoplasm of Connective and Soft Tissue
FS4:Malignant Neoplasm of Duodenum	FC7: Malignant Neoplasm of Rectum
FS5: Malignant Neoplasm of Colon	FC9: Malignant Neoplasm of Rectum
FS6: Malignant Neoplasm of Pancreas	FC10: Malignant Neoplasm of Colon

Table 19: Demographic Data for Adipose Cachectic Cases and Weight-Stable Adipose Controls.

Gender (M/F) FS: 4/2; FC: 4/2 **Characteristics**	**FS: N=6 FC: N=6**	**Mean**	**SEM**	**Median**	**Range**	**P-Value**
Age	Cach. Cont.	58.67 55.50	6.677 4.387	56.00 58.00	44-83 41-66	0.7002
Hemoglobin	Cach. Cont.	115.5 118.2	4.0978. 356	117.0 121.0	103-125 90-143	0.7803
Creatinine	Cach. Cont.	64.40 66.83	8.035 6.300	59.00 64.50	41-84 49-94	0.8142
Urea	Cach. Cont.	5.940 4.100	1.520 0.5013	5.300 3.800	2.9-11.1 3.0-6.5	0.2451
Total protein	Cach. Cont.	52.20 61.50	3.555 5.620	50.00 64.00	46-66 42-79	0.2163
Albumin	Cach. Cont.	27.80 35.33	3.382 2.929	26.00 37.00	20-40 23-44	0.1248
WBC	Cach. Cont.	9.633 7.100	1.295 1.186	9.350 5.900	6.0-13.3 4.4-12.1	0.1797
CRP	Cach. Cont.	11.00 18.40	5.874 5.810	7.000 19.00	1-34 1-34	0.3966
Baseline BMI	Cach. Cont.	24.79 28.41	2.290 0.8553	25.27 28.66	15.57-30.86; 25.26-31.23	0.1692
Current BMI	Cach. Cont.	23.12 28.37	2.027 0.9097	23.61 28.58	14.81-28.96; 24.91-31.23	* 0.0397
Baseline weights	Cach. Cont.	70.10 78.07	5.168 2.843	73.50 75.50	45.00-81.00; 71.40-89.00	0.2066
Current weights	Cach. Cont.	65.48 77.93	4.748 2.883	68.45 75.30	42.80-76.00; 72.00-89.00	*0.0489
Amount of WT loss	Cach. Cont.	4.617 0.133	0.7241 0.2171	4.500 0.0000	2.200-7.000 (-0.6000)-1.000	*** 0.0001
% of WT Loss	Cach. Cont.	6.489 0.178	0.8185 0.2991	5.826 0.0000	4.558-9.589 (-0.8403)- 1.370	***< 0.0001

Table 20 compares weight and BMI changes between current and baseline within each group (paired t-test). The cachectic fat cases (FS) had a significant difference between current and baseline data while weight-stable fat controls (FC) had no significant change.

Table 20: Paired t-Tests for Weight and BMI Change for Adipose Biopsies.

Characteristics	FS/FC: N=6/6	Mean	SEM	Median	Range	P-Value
Weight FS (cachectic)	Baseline Current	70.10 65.48	5.168 4.748	73.50 68.45	45.00-81.00 42.80-76.00	**0.0014
BMI FS (cachectic)	Baseline Current	24.79 23.12	2.290 2.027	25.27 23.61	15.57-30.86 14.81-28.96	**0.0035
Weight FC (weight-stable)	Baseline Current	78.07 77.93	2.843 2.883	75.50 75.30	71.40-89.00 72.00-89.00	0.5659
BMI FC (weight-stable)	Baseline Current	28.41 28.37	0.8553 0.9097	28.66 28.58	25.26-31.23 24.91-31.23	0.6247

5.1.2 Discussion

Similar to the discussion in section 4.1.2, in the above demographic analysis, patients included in the study were chosen meticulously to be at the early stages of cachexia (i.e. 5 - < 10% weight loss). Our subjects had a mean percentage of weight loss of 6.5% (range 4.6-9.6 %). Since systemic inflammation measured via serum CRP may or may not be present in patients with cachexia [7], our subjects were included regardless of their CRP values (range 1-34, Table 19). However, the CRP values between cachectic cases (FS) and weight-stable controls (FC) were ensured to be not statistically significant; P= 0.325 in muscle.

Nutritional and nutrition-related biochemical markers were all ensured to be comparable in both groups to objectively exclude the possibility of malnutrition as the primary cause of weight loss.

5.2 Heat Map of RNA Sequencing Data

5.2.1 Results

Figure 5shows the heat map of differentially regulated genes between cachectic fat cases (FS) and weight-stable fat controls (FC). The figure shows separation between both groups but not as clear cut as that seen in skeletal muscle. The heat map shows a total of 365 significantly upregulated genes and 93 downregulated genes (P-value < 0.05; and FDR < 0.5).

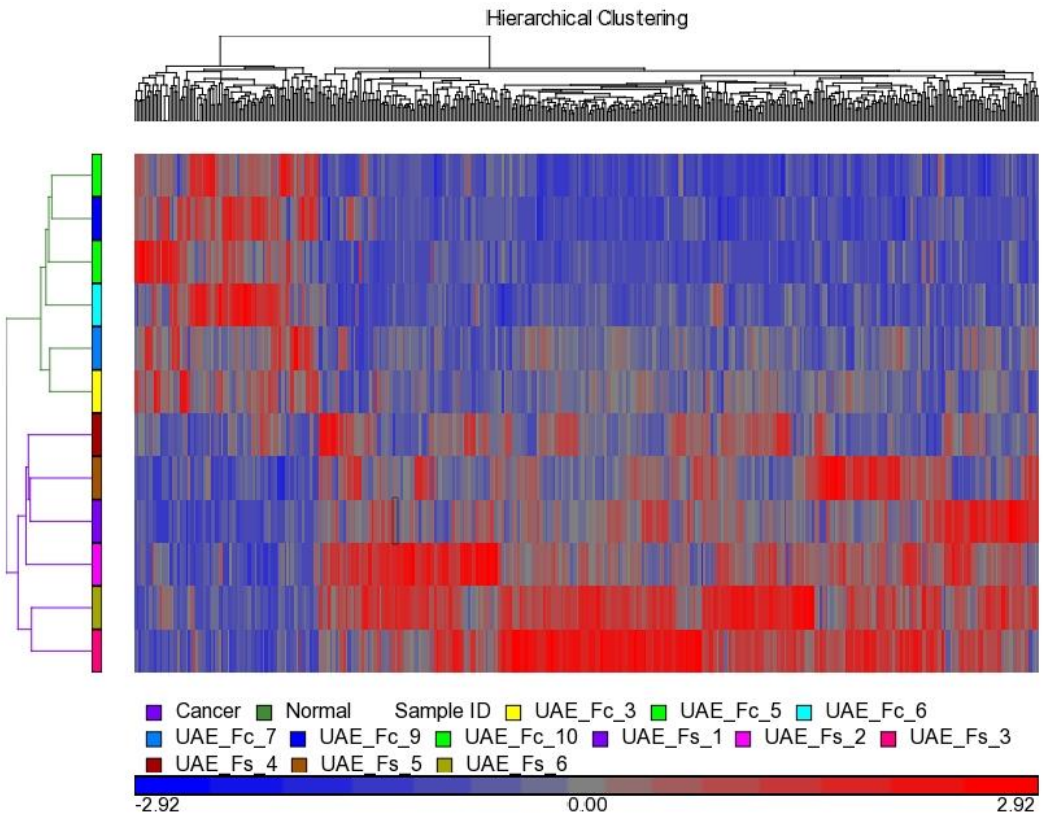

Figure 5: Heat Map Representation of the Differentially Expressed Genes in Adipose Tissue.

Gene expression is shown for the cachectic fat cases (FS; purple bars; lower panel) and weight-stable fat controls (FC; green bar; upper panel). Colors represent scaled and centered expression values: red represents higher expression, blue represents lower expression.

5.2.2 Discussion

This simple hierarchical cluster analysis revealed a clear visual distinction of gene expression signature between cachexia fat cases (FS) from those with weight-stable fat controls (FC).

5.3 Analysis of Differentially Expressed KEGG pathways

5.3.1 Upregulated KEGG Pathways in Cachectic Adipose Tissue

5.3.1.1 Results

Five KEGG pathways are differentially upregulated in cachectic fats cases vs. weight-stable control fats; Prion diseases, steroid biosynthesis, cytokine-cytokine receptor interaction, systemic lupus erythematosus (SLE), and N-Glycan biosynthesis. These pathways have 5, 3, 11, 6, and 4 significantly upregulated genes respectively (Table 21). Table 21 shows the individual genes in each pathway and the overall statistical significance for each of these pathways. The level of statistical significance for the prion disease pathway is an order of magnitude greater than that for the other pathways. Thus, it was the only discussed pathway as below. However, Table 22 shows a summary of the full gene name, fold change, and P-value for individual genes in each pathway.

Table 21: Differentially Upregulated KEGG Pathways in Cachectic Adipose Tissue.

KEGG Pathway	Gene	Gene	Gene	Gene	Gene	Gene	P-value	FDR
Prion diseases	BAX	C1QC	C6	LOC407835	NCAM2		0.0065	0.56
Steroid biosynthesis	EPB	SQLE	SOAT1				0.051	0.96
Cytokine-cytokine receptor interaction	CCR1	CCR5	CXCL10	CXCL11	IL1R1	IL20RA	0.056	0.91
	IL23A	MET	TGFB1	TNFRSF11A	TNFRSF8			
Systemic lupus erythematosus	FCGR1A	C1QC	C6	HIST1H3I	HLA-DMA	SNRPB	0.062	0.87
N-Glycan biosynthesis	ALG1	DPM2	MAN1B1	RPN1			0.077	0.87

Table 22: Data of Individual Genes in the Upregulated KEGG Pathways in Cachectic Adipose Tissue.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Fat-Up / Prion diseases			
BAX	BCL2-associated X protein	2.30	0.0272
C1QC	complement component 1, q subcomponent, C chain	3.24	0.0239
C6	complement component 6	2.26	0.0473
LOC407835	mitogen-activated protein kinase kinase 2 pseudogene	3.65	0.0246
NCAM2	neural cell adhesion molecule 2	2.27	0.0406
Fat-Up / Steroid biosynthesis			
EBP	emopamil binding protein (sterol isomerase)	2.34	0.0266
SQLE	squaleneepoxidase	2.45	0.0161
SOAT1	sterol O-acyltransferase 1	2.22	0.0222
Fat-Up / Cytokine-cytokine receptor interaction			
CCR1	chemokine (C-C motif) receptor 1	2.48	0.0234
CCR5	chemokine (C-C motif) receptor 5	2.10	0.0293
CXCL10	chemokine (C-X-C motif) ligand 10	4.31	0.0364
CXCL11	chemokine (C-X-C motif) ligand 11	5.84	0.0076
IL1R1	interleukin 1 receptor, type I	2.04	0.0339
IL20RA	interleukin 20 receptor, alpha	5.71	0.0094
IL23A	interleukin 23, alpha subunit p19	2.98	0.0389
MET	met proto-oncogene (hepatocyte growth factor receptor)	2.08	0.0307
TGFB1	transforming growth factor, beta 1	2.40	0.0235
TNFRSF11A	tumor necrosis factor receptor superfamily, member 11a, NFKB activator	3.35	0.0087
TNFRSF8	tumor necrosis factor receptor superfamily, member 8	2.96	0.0302
Fat-Up / Systemic lupus erythematosus (SLE)			
FCGR1A	Fc fragment of IgG, high affinity Ic, receptor (CD64); Fc fragment of IgG, high affinity Ia, receptor (CD64)	4.26	0.0118
C1QC	complement component 1, q subcomponent, C chain	3.24	0.0239
C6	complement component 6	2.26	0.0473
HIST1H3I	histone cluster 1, H3j; histone cluster 1, H3i; histone cluster 1, H3h; histone cluster 1, H3g; histone cluster 1, H3f; histone cluster 1, H3e; histone cluster 1, H3d; histone cluster 1, H3c; histone cluster 1, H3b; histone cluster 1, H3a; histone cluster 1, H2ad; histone cluster 2, H3a; histone cluster 2, H3c; histone cluster 2, H3d	4.14	0.0345
HLA-DMA	major histocompatibility complex, class II, DM alpha	2.03	0.0036
SNRPB	small nuclear ribonucleoprotein polypeptides B and B1	2.01	0.0205
Fat-Up / N-Glycan biosynthesis			
ALG1	asparagine-linked glycosylation 1, beta-1,4-mannosyltransferase homolog (S. cerevisiae)	2.24	0.0107
DPM2	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	2.52	0.0212
MAN1B1	mannosidase, alpha, class 1B, member 1	2.14	0.0460
RPN1	ribophorin I	2.02	0.0176

5.3.1.2 Discussion

Prion Diseases

BAX is a pro-apoptotic member of the Bcl-2 gene family. Apoptosis regulator BAX promotes apoptosis by binding to and antagonizing the Bcl-2 protein [253]. Activated BAX results in the release of cytochrome c and other pro-apoptotic factors from the mitochondria, often referred to as mitochondrial outer membrane permeabilization, leading to activation of caspases [254].

C6 gene codes for complement component 6 protein that is involved in the complement system. It is part of the membrane attack complex which can insert into the cell membrane and cause cell lysis to clear pathogens from an organism via a number of small proteins. The system is stimulated by one of several triggers causing release of proteases that cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. Thus results in massive amplification of the response and activation of the cell-killing membrane attack complex. Activation of BAX and C6 in adipose cells of cachectic patients could reflect their same functional properties prior to wasting of adipose tissue. NCAM2 encodes for one of three neural cell adhesion molecule (NCAM), also called CD56, is expressed on the surface of neurons, glia, skeletal muscle and natural killer cells.

5.3.2 Downregulated KEGG Pathways in Cachectic Adipose Tissue

5.3.2.1 Results

One KEGG pathway is differentially downregulatedin cachectic fat cases vs. weight-stable fat controls; the neuroactive ligand-receptor interaction. This pathway has 6 significantly downregulated genes and overall has a very high level of statistical significance (Table 23). Table 23 shows the individual genes in the pathway. Table

24 shows a summary of the full gene name, fold change, and P-value for individual genes in the neuroactive ligand-receptor interaction pathway.

Table 23: Differentially Downregulated KEGG Pathway in Cachectic Adipose Tissue.

KEGG Pathway	Gene	Gene	Gene	Gene	Gene	Gene	P-value	FDR
Neuroactive receptor ligand interaction	GABRG1	GRIA4	LEP	LHCGR	NMUR1	NPY1R	0.002	0.068

Table 24: Data of Individual Genes in the Downregulated KEGG Pathway in Cachectic Adipose Tissue.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Fat-Down / Neuroactive ligand-receptor interaction			
GABRG1	gamma-aminobutyric acid (GABA) A receptor, gamma 1	-3.26	0.0249
GRIA4	glutamate receptor, ionotrophic, AMPA 4	-3.10	0.0172
LEP	Leptin	-3.50	0.0032
LHCGR	luteinizing hormone/choriogonadotropin receptor	-3.90	0.0028
NMUR1	neuromedin U receptor 1	-2.69	0.0133
NPY1R	neuropeptide Y receptor Y1	-3.47	0.0242

5.3.2.2 Discussion

Neuroactive Ligand-Receptor Interaction Pathway

LEP encodes for leptin, a "satiety hormone" that signals energy sufficiency and thereby inhibits hunger. Leptin discovery was a breakthrough in 1994. It is secreted from adipocytes in proportion to fat stores and acts on receptors in the hypothalamus to regulate appetite [255]. Leptin suppresses feeding and decreases adiposity in part by release of αMSH and cocaine and amphetamine related transcript that directly suppress appetite, in part by inhibiting hypothalamic NPY synthesis and release, since NPY stimulates appetite, and partly by inhibiting release of agouti-related peptide that acts by blocking the action of αMSH at its receptor [256]. The regulation of appetite is a complex process involving multiple

hormones [257]. Hypothalamus is the main appetite regulator in the body that senses external multiple hormonal stimuli.

Leptin decreases food intake by affecting the balance between orexigenic and anorexigenic hypothalamic pathways [258]. Low leptin increases appetite and body weight. Crosstalk between leptin and inflammatory signaling known to be activated may be responsible for this paradox [258]. Many studies looked at the serum level of leptin in cancer patients. Cachectic esophageal cancer patients had decreased circulating leptin [259-262].

Interestingly, *in vitro* and *in vivo*, insulin plays a major transcriptional activation that stimulates leptin circulation level [263]. However, patients with cachexia do usually have high insulin resistance which does not elevate, at least in our study, the adipocyte production of leptin. This downregulation of leptin production in adipocyte does not seem to increase appetite in cancer patient. This reflects that the deregulation of appetite and weight in cachectic patient is a complex process.

Leptin levels are low, as expected, signaling that there are NOT sufficient energy stores in fat. However, this is not reflected in the expected increase in appetite! Paradoxically, NPY expression in the ARC is decreased (you would expect it to be increased in the absence of leptin signaling) and that of POMC (source of αMSH) increased (you would expect it to be decreased in absence of leptin). There is speculation that this is a response to GAD15 in the hypothalamus and GAD15 expression was significantly increased in cachectic muscle in the present study [258].

Leptin level are negatively correlated with the degree of cachexia [259]. Although regulation of fat stores is deemed to be the primary function of leptin, obese

individuals have a decreased sensitivity to leptin occurs, resulting in an inability to detect satiety despite high energy stores [264].

LHCGR codes for the luteinizing hormone/choriogonadotropin receptor protein. LHCGR is abundantly expressed in preadipocytes and mature adipocytes [265]. It is studied the most in the gonads. In the ovary, the LHCGR is required for follicular maturation and ovulation, as well as luteal function upon hormonal stimulation [266]. Low levels of the receptor are repeatedly linked to infertility.

While the function of this receptor is uncharacterized in adipose tissue, we do believe there is a strong correlation between the downregulation of that receptor and the depletion of fat stores. Female athletes one of the commonest examples of infertility caused by depletion of fat stores which both could be possibly due, at least in part, to the downregulation of the receptor in gonads and adipose tissue.

NPY1R codes for the Neuropeptide Y receptor type 1 protein. NPY, the neuropeptide Y, is a neurotransmitter involved in food intake and body weight regulation [267] and prevention of lipolysis [268] via Y1 and Y5 receptors. Y1 receptors are coded by NPY1R which is increased in adipose tissue samples of obese patients and in turn signals for inhibition of lipolysis [269]. Additionally, NPY1R expression in visceral adipose tissue is positively correlated with body weight, BMI, and insulin levels [267]. Downregulation of the gene in cachectic adipose tissue neutralizes its "lipolysis inhibition" signal which may contribute to adipose wasting.

NMUR1 codes forneuromedin-U (NMU) receptor 1. NMU overexpressing tumors in mice resulted in lowering of body weight and cachexia [270]. NMUR1 in human is highly expressed in intestinal adipose tissue [271].

Intracerebroventricular administration of NMU markedly suppresses food intake in rats [272]. The downregulation of NMUR1 could be a compensatory mechanism to oppose the possible outcome of receptor stimulation with is documented to lead to cachexia in mice.

GABRG1 encodes for gamma-aminobutyric acid receptor subunit gamma-1 protein which is a subunit of the GABAA receptor. GABAA receptors are located synaptically as well as presynaptically and are activated by gamma-aminobutyric acid (GABA) [273]. GABA is a neuroinhibitory transmitter in the brain [273]. Oral GABA given activates peripheral GABA receptors and this improves glucose tolerance, improves insulin sensitivity, and prevents obesity without altering calorie intake in mice [274]. Thus, the downregulation in cachectic adipose tissue could play a role in insulin resistance seen in cachectic cancer patients and could be a compensatory measure trying to stimulate weight gain.

GRIA4 encodes for glutamate receptor 4. It is a member of a family of L-glutamate-gated ion channels that mediate fast synaptic excitatory neurotransmission. Glutamate receptors are protein complexes known to be the main excitatory neurotransmitter receptors in the mammalian brain [275]. The regulation of glutamate receptors is influenced by leptin levels [276] which is also downregulated in cachectic adipose tissues.

Surprisingly, five out of the six above genes are receptors. The sixth gene is leptin, which also functions to downregulate one of receptors (i.e. GRIA4) is also downregulated. Most of these receptors are well studied and characterized in the brain, while this is the first time they are reported, as far of our knowledge, to be collectively downregulated in human adipose tissue.

5.4 Analysis of Highly Significantly Altered Genes in Cachectic Adipose Tissues

In visceral fat, 16 genes showed highly significant change in expression (10 upregulated and 6 downregulated: P<0.001-0.0002, FDR 0.5).

5.4.1 Highly Significantly Upregulated Genes

5.4.1.1 Results

Table 25 shows the 10 highly significantly upregulated genes in cachectic fat (P<0.001-0.0002; DFR 0.5). They include 2 involved with transcriptional regulation, 2 transporters, on in each of the development, angiogenesis inhibitor, metabolism, and signaling, and 2 uncharacterized.

Table 24 categorizes individual gene into respective functional domain. It also shows the full gene name, fold change, and P-value.

Table 25: Highly Significantly Upregulated Genes in Cachectic Adipose Tissue.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Transcription regulation			
RPP40	RibonucleaseP protein subunit p40	3.49	0.001
IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2	2.49	<0.001
Transporters			
SLC19A1	Solute carrier family 19 (folate transporter), member 1	2.34	0.001
STEAP3	Metalloreductase STEAP3	2.57	0.0002
Development			
IFRD2	interferon-related developmental regulator 2	2.25	0.001
Angiogenesis Inhibitor			
BAI2	Brain-specific angiogenesis inhibitor 2	4.43	0.0008
Metabolism			
PUSL1	pseudouridylate synthase-like 1	2.37	0.001
Signaling			
LYPD1	Ly6/PLAUR domain-containing protein 1	5.54	0.001
Un-characterized			
CCDC94	Coiled-coil domain containing 94	2.51	0.0003
C15orf57	chromosome 15 open reading frame 57	2.49	0.001

5.4.1.2 Discussion

Transcription Regulation

RPP40 encodes for ribonuclease P protein subunit p40, which generates mature tRNA molecules by cleaving the 5'-end of the leader sequence of precursor tRNA. RNase P is a ribozyme; a unique form of RNase that functions as a catalyst in the same way that a protein enzymes work [277] and is required for normal and efficient transcription of various small noncoding RNAs, such as tRNA, 5S rRNA, SRP RNA and U6 snRNA genes [278, 279]. However the RNase P in human mitochondria is a protein with no RNA component. Moreover, the enzyme is composed of a tRNA methyltransferase, a short-chain dehydrogenase/reductase-family member, and a protein of hitherto unknown functional and evolutionary origin, possibly representing the enzyme's metallonuclease moiety [280].

IGF2BP2 encodes insulin-like growth factor 2 mRNA-binding protein 2, a member of the IGF-II mRNA-binding protein family [281]. It functions by binding to the 5' UTR of the insulin-like growth factor 2 mRNA and regulates its translation by modulating the rate and location at which it encounters the translational apparatus and shields it from endonuclease attacks or microRNA-mediated degradation [281]. An important paralog of this gene is IGF2BP3 [281]. There is a strong association between IGF2BP2 insulin resistance and type 2 diabetes mellitus, however, the exact mechanisms has not been determined [282-285].

On the other hand, adipose tissue plays a role in secreting a variety of factors which are involved in the modulation of adipose mass [286]. Adipose tissue development and metabolism are regulated by growth hormone (GH) and the insulin-like growth factor (IGF) system [286]. GH plays an anti-insulin effect; however, the mechanism by which GH antagonizes insulin stimulation of lipogenesis is unknown [286].

IGF2BP2 downregulates the expression of IGF2; a growth factor that plays a pivotal role in controlling adipogenesis [287]. Therefore, IGF2BP2 may contribute to type 2 diabetes through alterations in adipose tissue [287]. Also, a more than two-fold increase in IGF2BP2 expression level was observed in the adipose tissue of diabetic patients compared to controls [288]. Similarly, an altered expression of IGF2BP2 in adipocytes of subjects with type 2 diabetes compared with healthy people was also detected [289]. Thus, an increase in the IGF2BP2 in the adipose tissue of cachectic subject in our study is no surprise since it alters adipogenesis and promotes insulin resistance.

Transporters

SLC19A1 encodes for solute carrier family 19 (folate transporter), member 1 protein. It facilitates intracellular uptake of reduced folate to maintain intracellular concentrations of folate. Despite of the plethora of studies on SLC19A1, the majority of these investigated it in terms of polymorphic effects on drug dosing (i.e. the antimetabolite; methotrexate). Thus, studies investigating the role of this transporter in adipose tissues are lacking. However, SLC19A1 has three protein kinase C (PKC) phosphorylation sites [290]. Effect of PKC is cell type specific. In adipose tissue, PKC transduces the effects of adrenergic agonists (specifically agonists of the β3 receptor). β3-adrenergic agonists stimulate brown adipose tissue thermogenesis [291]. In white adipose tissues; however, β3-adrenergic agonists increase energy expenditure and lipolysis, which in turn promote lipid mobilization [291]. These effects causes adipose tissue wasting and decreased fat stores [291] which are all key manifestations in the cachectic patients. β3-Adrenergic agonists also enhance glucose disposal to provide energy for the maintenance of metabolic activity and muscle function under limited energy availability [291]. Other adrenoreceptors have

other effects on lipid and glucose metabolism; α1-adrenoceptors and β2-adrenoceptors are linked to glycogenolysis and gluconeogenesis in adipose tissue; while β1-adrenoceptors and β3-adrenoceptorsare linked to lipolysis in adipose tissue [292].

STEAP3 codes for six-transmembrane epithelial antigen of prostate STEAP family member 3, a metalloreductase that is capable of converting iron from an insoluble ferric (Fe^{3+}) to a soluble ferrous (Fe^{2+}) form. This family comprises STEAP1-4; all of which share metalloreductases activity [293]. They participate in a wide range of biologic processes, such as molecular trafficking in the endocytic and exocytic pathways and control of cell proliferation and apoptosis [293]. STEAP3 is also known as tumor-suppressor activated pathway-6 (TSAP6) or dudulin-2. Mutations in STEAP3 result in an autosomal recessive trait characterized by an inefficient supply of iron to erythrocytes, leading to anemia and iron overload [294].

STEAP3 is upregulated in white adipose tissue of rats fed high fat diet and significantly downregulated in rats treated with capsaicin (a potential anti-obesity drug) [295]. This indicate that STEAP3 increases when fat supply is abundant and not during times of energy deficit. It is possible that STEAP3 increases as a compensatory mechanism to signal resistance to fat loss and/or to stimulate energy storage that mimic the state of abundance under the forces or triggers of wasting.

On the other hand, one anti-obesity strategy is to block adipocyte differentiation, preventing fat storage and the secretion of adipokines [296]. STEAP3 is upregulated in differentiating adipocytes [296]. This suggests that the adipose tissue in cachexia is undergoing an early-onset protective mechanism by inducing adipocyte differentiation in an attempt to prevent further lipolysis and wasting.

Development

IFRD2 codes for interferon-related developmental regulator 2. Very little is known, so far, about I the FRD2 gene. However another member of this gene family, IFRD1 is expressed in skeletal and cardiac muscle. It is also expressed in differentiating myoblasts in vitro [297]. IFRD1 knockout mice have delayed muscular regeneration after muscle crush damage [298]. In contrast, upregulation of IFRD1 in injured muscle potentiates muscle regeneration [299].

IFRD1 was also recently studied in white adipose tissue in obese mice, where its expression is increased [300]. It is also upregulated under stressful stimuli such as hypoxia [300], where it prevents the inhibition of adipogenesis [300]. This effect is abrogated by knockdown of IFRD1 by shRNA which inhibits adipogenesis. These findings suggest that IFRD1 may play a role in adipogenesis under conditions of hypoxia and obesity.

The uniqueness of importance of IFRD1 in muscle regeneration and adipogenesis, suggests a similar mechanism of action for IFRD2, which may be more specific for adipose tissue. Furthermore, since IFRD1 potentiates the regenerative process, IFRD2 might be activated in cachectic adipose tissue as a defense mechanism to reduce the loss of adipocytes due to wasting.

Metabolism

PUSL1 encodes for pseudouridylate synthase-like 1, an uncharacterized metabolism protein with pseudouridine synthase activity. Pseudouridylate synthase is an enzyme that catalyzes the conversion of uracil and D-ribose 5-phosphate to pseudouridine 5'-phosphate and H_2O. This enzyme belongs to the family of lyases that participate in pyrimidine metabolism; an indicator for the rate of DNA turnover

[301]. Despite not knowing exactly how the PUSL1 enzyme functions in adipose tissue, what we know so far about pyrimidine metabolism is extremely exciting. All pyrimidine nucleotides are synthesized *de novo*, mainly in the liver. Pseudouridine is an intermediate in the pyrimidine synthesis pathway. Pseudouridylate synthase activity allows E. coli to grow in the presence of pseudouridine, with growth paralleling measured enzymic activity [302]. More excitingly, pseudouridylate synthase is a catabolite repressible enzyme [303]. Catabolite repression, usually observed in bacteria and other microorganisms, is the inhibition of synthesis of enzymes involved in catabolism of carbon sources other than the preferred one [304].

The above data can be correlated to cachectic patients whose adipose tissues is trying to repress catabolism and as a compensatory mechanism, rather trying to build up the key building blocks of adipogenesis (i.e. ribonucleic acid material or pyrimidines). With pyrimidines being mostly synthesized in the liver; it is not surprising that unlike the atrophying livers of patients losing weight due to starvation, the livers of patients with cachexia become significantly enlarged, probably due to overload.

Signaling

LYPD1 encodes for Ly6/PLAUR domain-containing protein 1. However, the understanding of this protein in the context of cachexia is hard since very little, if any, is known about it. LYPD1 partial silencing results in a highly significant increase in overall anchorage-independent growth in HeLaHF cells. LYPD1 overexpression reduced cell survival and anchorage-independent growth and induced higher levels of apoptosis in soft agar [305].

LYPD1 is associated with bannayan-riley-ruvalcaba syndrome, a rare overgrowth syndrome [306]. One of the predominant features of the syndrome is the occurrence of multiple subcutaneous lipomas [306, 307].

Angiogenesis Inhibitor

BAI2 encodes for brain-specific angiogenesis inhibitor 2, a member of the adhesion-G-protein coupled receptors (adhesion-GPCRs) family of receptors [308]. BAI2 and BAI3 are similar to BAI1, have similar tissue specificities and structure and may also play a role in angiogenesis [309]. BAI2 is dominantly expressed in the brain [310]. However, its functions are still unclear but it is suggested that BAI2 is a functional GPCR regulated by proteolytic processing and activates the NFAT pathway [310]. It is known that the nuclear factor of activated T cell (NFAT) group of transcription factors contributes to glucose and insulin homeostasis [311]. Expression of NFATc2 and NFATc4 is induced upon adipogenesis and in obese mice, while ablation of both NFATc2 and NFATc4 increases insulin sensitivity [311].

If BAI2 upregulation is confirmed to activate the NFAT pathway, this suggests that such activation signals induced adipogenesis as an adaptive response to the wasting triggers in cachexia. Also, upregulation of this pathway could explain, at least in part, by the increased insulin resistance in these patients.

5.4.2 Highly Significantly Downregulated Genes in Cachectic Adipose Tissues

5.4.2.1 Results

Table 26: Highly Significantly Downregulated Genes in Cachectic Adipose Tissue.

Gene Symbol	Gene Full Name	Fold Change	P-Value
Metabolism			
CES1	Liver carboxylesterase 1	-5.65	0.0006
NQO1	NAD(P)H dehydrogenase [quinone] 1	-3.82	<0.0006
Transcription Factor			
AFF3	AF4/FMR2 family member 3	-2.30	0.00003
Angiogenesis Inhibitor			
TNMD	Tenomodulin	-15.46	<0.0007
Uncharacterized			
LOC100996634	Transmembrane protein FLJ37396	-15.24	0.0001
TRIM16L	Tripartite motif containing 16-like	-2.98	<0.0002

5.4.2.2 Discussion

Metabolism

CES1 codes for the liver carboxylesterase 1 enzyme. CES1 is present in most tissues with higher levels in the liver [312], and is highly regulated in adult adipose tissue [313], with higher levels in obese subjects and lower levels during weight loss [314]. This enzyme hydrolyzes long-chain fatty acid esters and thioesters. CES1 is an inducible serine esterase that hydrolyses many drugs (to activate or inactivate them) and a nutrients, as well as cholesterol esters, etc. CES1 could be an important player in metabolism of triglycerides and cholesterol in adipocytes [315]. Thus, CES1 is suggested to play a major role in the trafficking of lipids and lipid metabolism in hepatocytes and its upregulation mediates adipocyte lipolysis through the hydrolysis of cholesteryl esters and triglycerides [313]. Studies correlated

increased expression to be a measure of adiposity and insulin resistance (well here we have decreased adiposity but insulin resistance.

Expression level of CES1 in adipose tissue was positively associated with body mass index, homeostasis model assessment-insulin resistance, and level of fasting glucose, insulin, and triglycerides [315]. In murine adipocytes, CES3 was identified as a gene that increased lipid storage [316]. All of which are measures and/or triggers of adiposity.

In vitro and *in vivo* studies suggested that inhibition of CES1 could help treat type 2 diabetes [316]. In murine models of diabetes, a CES3 inhibitor protected animals from weight gain, decreased liver lipid accumulation and increased insulin sensitivity [316]. CES1 activity was upregulated in adipose tissue from patients with type 2 diabetes, compared with that in lean controls [316], and also upregulated in obese children [313].

Hence, the highly significantly downregulated CES1 in the adipose tissue of our cachectic patients reflects the actual weight losing state of our patients, and explain the compensatory state of adipose tissue to resist lipolysis and the state of insulin resistance.

NQO1 codes for an NAD(P)H dehydrogenase [quinone] 1 enzyme which is a member of family of enzymes that prevents the one electron reduction and detoxification of quinones and their derivatives that results in the production of radical species [317]. NQO1 has a high expression in human adipose tissue, particularly in large adipocytes and is reduced by weight loss (similar to CES1) and also correlates with adiposity [318].

NQO1 regulates the ubiquitin-independent p53 degradation pathway through stabilization of p53. NQO1 downregulation reduces p53 stability, leading to resistance to some drugs including chemotherapeutics. One important and exciting correlation is the increased hematotoxicity in benzene-exposed workers in China with a certain NQP1 genotype isolated from bone marrow that showed failure to express NQO1 [319]. NQO1 null mice exhibited increased toxicity when administered menadione [320]. However, these mice exhibit significantly lower levels of abdominal adipose tissue, lower blood levels of glucose, no change in insulin, and higher levels of triglycerides, b-hydroxy butyrate, pyruvate, lactate, and glucagon and insulin resistant as compared with wild-type mice [317]. Also, the liver glycogen reserve was found to be decreased [317]. All of which data suggest that the loss of NQO1 causes alteration in the intracellular redox activities leading to reduction in pyridine nucleotide synthesis and reduced glucose and fatty acid metabolism resulting in a significant reduction in the amount of abdominal adipose tissue [317]. In addition, the loss of NQO1 leads to the alteration in lipid deposition due to the decrease in gluconeogenesis and fatty acid metabolism [321].

NQO1 mutations are also found to be associated with tardive dyskinesia (TD) an incurable form of dyskinesia; a disorder resulting in involuntary, repetitive, and tardive (slow or belated onset) body movements [322].

Transcription Factor

AFF3 is an AF4/FMR2 family member 3 that encodes a tissue-restricted nuclear transcriptional activator [323] that functions in lymphoid development and oncogenesis [324, 325]. AFF3 is associated with kidney disease in diabetics with type 1 [326] but not type 2 diabetics [327].

Angiogenesis Inhibitor

TNMD codes for tenomodulin; a novel chondromodulin-I related gene. Chondromodulin-I is a cartilage-specific glycoprotein that functions to stimulate chondrocyte growth and to inhibit tube formation of endothelial cells [328]. The TNMD transcript is found in hypovascular tissues such as tendons and ligaments and that is believed to be an angiogenesis inhibitor [329]. TNMD is also expressed in both the adipocyte and stromal vascular fraction of adipose tissue [330].

TNMD expression is decreased in the adipose tissue of subjects who have lost weight and its expression level is strongly correlated with body mass index [330]. Expression remained was also consistently remained downregulated after weight loss of subjects on a low-calorie diet and after weight maintenance [331]. TNMD is associated the risk of developing diabetes [332], although this association is not yet well-understood. However, genetic variation of the TNMD gene is associated with serum levels of systemic immune mediators which have a well-established connection to obesity, metabolic syndrome, and type 2 diabetes [333].

The potential mechanisms that links TNMD with the pathogenesis of obesity, diabetes, and metabolic syndromes are yet to be confirmed [330]. However, in the context of our weight losing cachectic patients, TNMD downregulation is perhaps expected. However, the 15-fold downregulation is massive given the small degree (5-10%) weight loss. Thus, we believe this dramatic downregulation is important given the effect of weight loss, insulin resistance, and the metabolic syndrome of cachexia.

5.5 Anticipated Results from Previous Studies

5.5.1 Preclinical Findings

Adipose depletion in cachexia is a function of lipolysis [334]. Adipose tissue atrophy starts with the excessive hydrolysis of triglycerides to free fatty acids and glycerol that is then mobilized and released in the circulation. The key enzymes catalyzing this reaction are the hormone sensitive lipase (LIPE) and adipose triglyceride lipase (PNPLA2) [335]. Preclinical studies showed that inhibition of lipolysis through genetic ablation of LIPE or PNPLA2 ameliorates certain features of murine model of cancer cachexia [336]. Expressions of neither LIPE nor PNPLA2 were significantly altered in the present study. However, a different lipid mobilizing factor; zinc-α glycoprotein 1(AZGP1), has been previously shown to be involved in cachexia as a clinical finding [337]. Expression of AZGP1 was also increased in the present study but with only borderline significance (Fold change 2.79, P-value 0.0576).

Parathyroid hormone-related protein (PTHLH) is a causative factor of hypercalcemia in malignancy. A recent suggested hypothesis to cancer cachexia is that there is adipose tissue "browning" that results in an increased in energy expenditure leading and greater thermogenesis [338]. In murine model cancer cachexia, PTHLH induced the expression of genes involved in thermogenesis in adipose tissues while blocking PTHLH blocked adipose tissue browning [339]. The present study revealed no change in expression of either PTHLH or its associated receptor, PTH1R suggesting that tissue browning theory that mediates thermogenesis and consequently an increase in energy expenditure is not the one of the contributing factors in human subjects.

5.5.2 Clinical Findings

In recent years, adipose tissue is been recognized to have major endocrine functions [340]. Several pro-inflammatory cytokines and hormones are believed to be produced in cancer cachexia patients, at least in part by the adipose tissue. Cytokines (TNF, IL6, and IFNG) and hormones (such as leptin) have been investigated in the context of cancer cachexia. Leptin is the "satiety hormone" made by adipose cells that helps in the regulation of energy balance by suppressing appetite [255]. In obese individuals, however, the body has a decreased sensitivity to leptin resulting in an inability to detect satiety despite of the high energy stores [264]. Clinical studies found that leptin levels are significantly lower in cachectic patients [259, 261, 341] while higher levels of IL6 were associated with future development of cachexia [341].

The present study shows a significant reduction in leptin expression as expected (Fold change -3.50, P-value 0.0032). This suggests that leptin does not play an important role in cancer cachexia development but rather an indicator for depleting adipose stores in these patients. On the other hand, expression of IL6 was elevated but not statistically significant. However, it is possible that IL6 expression is increased in some but not all cachectic cancer patients.

5.6 Verified Results Using Real-Time Reverse Transcription PCR

Despite of the high reliance of the quantitative capability of the RNA sequencing results, it is important to verify part of the expression results using a different method. A cherry-picked selection of genes that are of a high significance and high fold-change were picked. Confirming the difference of high fold-change expression data can allow us to check and verify them later-on at protein level. One upregulated

gene; CXCL11, and three downregulated genes; TNMD, LEP, and LHCGR were confirmed.

5.6.1 Results

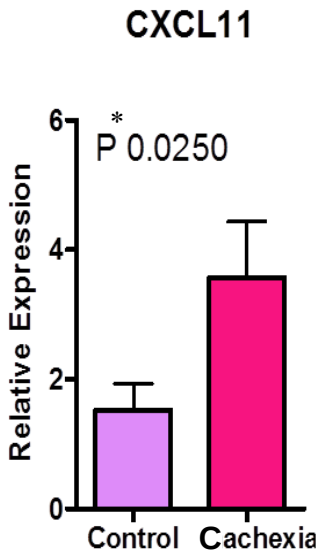

Figure 6: Upregulation of Expression of Selected Genes Confirmed by Real-Time RT-PCR in Cachectic Adipose Tissue.

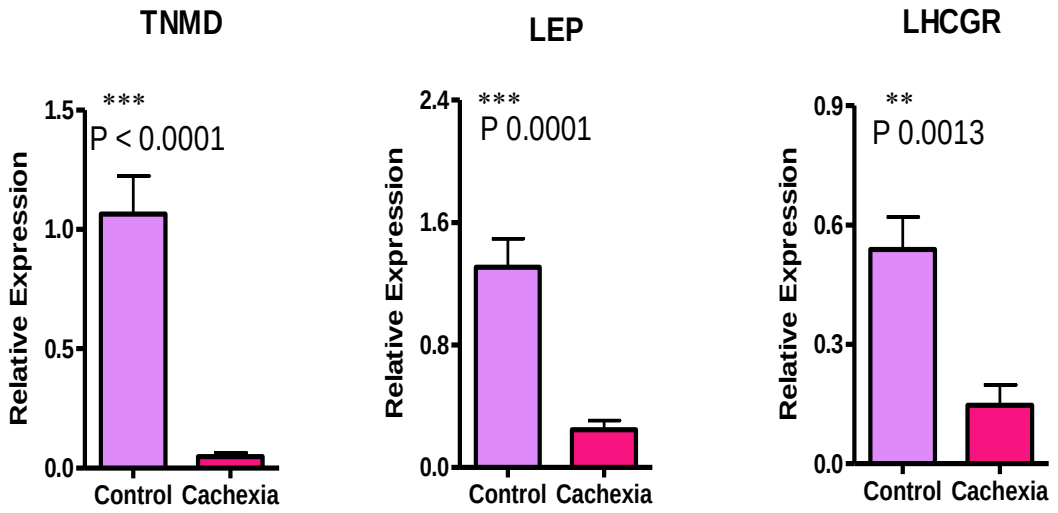

Figure 7: Downregulation of Expression of Selected Genes Confirmed by Real-Time RT-PCR in Cachectic Adipose Tissue.

All chosen genes that were previously shown to be upregulated/downregulated were reproducible via real-time RT-PCR (Figure 6 and 7).

5.6.2 Discussion

Expressions of one upregulated gene; CXCL11 that is statistically significant was confirmed via real-time RT-PCR.

CXCL11 codes for chemokine (C-X-C motif) ligand 11 protein (Table 22). It is a small cytokine that belongs to the CXC chemokine family that is also called interferon-inducible T-cell alpha chemoattractant (I-TAC) and Interferon-gamma-inducible protein 9 (IP-9)[342]. Normally, expression of CXCL11 in mice adipose tissue was found to be consistently very low [343]. Adipose tissue macrophages (ATM), which are tissue resident macrophages, are present in adipose tissue. It was found that these ATMs are transiently recruited to adipose tissue at times of acute weight loss [344, 345]. ATM was found to have a high level expression of CXCL11 [346]. So far, the right justification of elevated levels of CXCL11 in adipose tissue is

not reached. However, we know that their expression level is normally downregulated.

The confirmed downregulated genes were TNMD, LEP, and LHCGR. The TNMD (section 5.4.2.2) is an angiogeneic inhibitor gene that codes for tenomodulin; a novel chondromodulin-I related gene. TNMD is expressed in both the adipocyte and stromal vascular fraction of adipose tissue [330]. TNMD expression is decreased in the adipose tissue of subjects who have lost weight and its expression level is strongly correlated with body mass index [330]. Expression remained was also consistently remained downregulated after weight loss of subjects on a low-calorie diet and after weight maintenance [331]. TNMD is associated the risk of developing diabetes [332], although this association is not yet well-understood. However, genetic variation of the TNMD gene is associated with serum levels of systemic immune mediators which have a well-established connection to obesity, metabolic syndrome, and type 2 diabetes [333].

The potential mechanisms that links TNMD with the pathogenesis of obesity, diabetes, and metabolic syndromes are yet to be confirmed [330]. However, in the context of our weight losing cachectic patients, TNMD downregulation is perhaps expected. However, the 15-fold downregulation is massive given the small degree (5-10%) weight loss. Thus, we believe this dramatic downregulation is important given the effect of weight loss, insulin resistance, and the metabolic syndrome of cachexia.

LEP (as discussed in section 5.3.2.2) encodes for leptin, a "satiety hormone" that signals energy sufficiency and thereby inhibits hunger. Leptin discovery was a breakthrough in 1994. It is secreted from adipocytes in proportion to fat stores and acts on receptors in the hypothalamus to regulate appetite [255]. Leptin suppresses

feeding and decreases adiposity in part by release of αMSH and cocaine and amphetamine related transcript that directly suppress appetite, in part by inhibiting hypothalamic NPY synthesis and release, since NPY stimulates appetite, and partly by inhibiting release of agouti-related peptide that acts by blocking the action of αMSH at its receptor [256]. The regulation of appetite is a complex process involving multiple hormones [257]. Hypothalamus is the main appetite regulator in the body that senses external multiple hormonal stimuli.

Leptin decreases food intake by affecting the balance between orexigenic and anorexigenic hypothalamic pathways [258]. Low leptin increases appetite and body weight. Crosstalk between leptin and inflammatory signaling known to be activated may be responsible for this paradox [258]. Many studies looked at the serum level of leptin in cancer patients. Cachectic esophageal cancer patients had decreased circulating leptin [259-262].

Interestingly, *in vitro* and *in vivo*, insulin plays a major transcriptional activation that stimulates leptin circulation level [263]. However, patients with cachexia do usually have high insulin resistance which does not elevate, at least in our study, the adipocyte production of leptin. This downregulation of leptin production in adipocyte does not seem to increase appetite in cancer patient. This reflects that the deregulation of appetite and weight in cachectic patient is a complex process.

Leptin levels are low, as expected, signaling that there are NOT sufficient energy stores in fat. However, this is not reflected in the expected increase in appetite! Paradoxically, NPY expression in the ARC is decreased (you would expect it to be increased in the absence of leptin signaling) and that of POMC (source of αMSH) increased (you would expect it to be decreased in absence of leptin). There is speculation that this is a response to GAD15 in the hypothalamus and GAD15

expression was significantly increased in cachectic muscle in the present study [258].

Leptin level are negatively correlated with the degree of cachexia [259]. Although regulation of fat stores is deemed to be the primary function of leptin, obese individuals have a decreased sensitivity to leptin occurs, resulting in an inability to detect satiety despite high energy stores [264].

LHCGR (as discussed in section 5.3.2.2) codes for the luteinizing hormone/choriogonadotropin receptor protein. LHCGR is abundantly expressed in preadipocytes and mature adipocytes [265]. It is studied the most in the gonads. In the ovary, the LHCGR is required for follicular maturation and ovulation, as well as luteal function upon hormonal stimulation [266]. Low levels of the receptor are repeatedly linked to infertility.

While the function of this receptor is uncharacterized in adipose tissue, we do believe there is a strong correlation between the downregulation of that receptor and the depletion of fat stores. Female athletes one of the commonest examples of infertility caused by depletion of fat stores which both could be possibly due, at least in part, to the downregulation of the receptor in gonads and adipose tissue.

Chapter 6: Overlapping Genes in Skeletal Muscle and Adipose Tissue

This chapter aims at looking at the significantly altered gene expressions that are commonly altered in both; muscle and adipose tissues.

6.1 Upregulated Genes

6.1.1 Results

Table 27: Upregulated Overlapping Genes.

Gene Symbol	Gene Full Name	Muscle		Fat	
		Fold change	P-Value	Fold change	P-Value
ASS1	Argininosuccinate Synthase 1	3.70	0.0064	2.14	0.0323
CLCN2	Chloride Channel, Voltage-Sensitive 2	9.48	0.0163	2.29	0.0239
PGAP3	Post-GPI Attachment To Proteins 3	2.80	0.0032	2.12	0.0066
RPS10	Ribosomal Protein S10	4.58	0.0045	2.18	0.0256
SLC36A1	Solute Carrier Family 36 (Proton/Amino Acid Symporter), Member 1	4.00	0.0039	2.07	0.0323
TGM2	Transglutaminase 2	2.20	0.0114	2.42	0.0244

6.1.2 Discussion

ASS1 is an argininosuccinate synthase enzyme that catalyzes the penultimate step of the arginine biosynthetic pathway. Arginine is classified as a semi-essential or conditionally essential amino acid, depending on the developmental stage and health status of the individual [347]. Arginine biosynthesis is only needed in unhealthy individuals because there are not getting enough nutritional supply. It serves many important roles such as cell division, wound healing, ammonia metabolism, immune function, and the release of hormones [347, 348]. The direct correlation of argininosuccinate synthase function in skeletal and adipose tissues in cachectic individuals is unknown. It can be stipulated that in both tissues (when

combined, they constitute the majority of human body mass), cachexia is a huge insult requiring healing and repair (probably), and definitely replacement.

On the other hand, mutations in the ASS1 gene cause type I citrullinemia because of alteration of an important step of the urea cycle [349]. Individuals with type I citrullinemia develop symptoms when alteration in the enzyme causes ammonia build up in the body. Hyperammonemia is significantly higher in terminally ill cancer patients [350]. Symptoms of hyperammonemia in type I citrullinemia includes lack of energy (lethargy), poor feeding, vomiting, and others. All of which are seen in cachexia. Skeletal muscle plays a role in ammonia detoxification. Ammonia is also known to stimulate the hypothalamic satiety centers which suppress appetite and lead to cachexia [351]. Generally, L-arginine reduces adiposity, increase muscle mass, and improve the metabolic profile in animals and humans [352]. All of which are important adaptive requirement in cachectic patients.

CLCN2 codes for chloride channel protein 2 that is one of 13 members in the chloride channel superfamily of poorly understood ion channels that are implicated in many cellular processes. Chloride channels in skeletal muscle play a role in setting and restoring the resting membrane potential, thus preventing muscle stiffness or myotonia [353]. Mutations in the human skeletal muscle chloride channel are associated with dominant and recessive congenital myotonia [353]. While such a role is clearly correlated to muscle regulation, the reason of the upregulation in adipose tissue is yet to be understood.

PGAP3 codes for post-GPI attachment to proteins 3 that encodes a glycophosphatidylinositol (GPI)-specific phospholipase A2 that is expressed in the Golgi apparatus. The enzyme is involved in fatty acid GPI remodeling that is critical for proper association between GPI-anchored proteins and lipid rafts [354]. In

muscle, mutation in PGAP3 causes muscular hypotonia [354]. PGAP3 is responsible for the modification of the fatty acid residues on the GPI anchor in a maturation process that occurs in the endoplasmic reticulum and Golgi [354].

RPS10 is another important gene in the cachectic organs that is likely to be involved in the adaptive responses to the forces of wasting. RPS10 codes for the 40S ribosomal protein S10 that is one of the two subunits making the ribosomes; the organelles that catalyze protein synthesis [355]. Also, RPS10 expressed sequence tags were isolated from the skeletal muscle of neonatal healthy and splay leg piglets [356]. It belongs to the S10E family of ribosomal proteins. RPS10 expression was reduced in brown adipose tissue of hibernating arctic ground squirrels compared to mice [357]. Hibernation is an energy-saving strategy adopted by a wide range of mammals to survive highly seasonal or unpredictable environments.

SLC36A1 codes for a solute carrier family 36 (a proton-coupled amino acid transporter (PAT1) protein, that mediates symport of protons and small neutral amino acids. This carrier has a role in absorption of amino acids from luminal protein digestion of the brush border membrane driving transport of amino acids into the cytosol (presumably in the gut during absorption) [358]. These amino acids, in turn, activate mTORC1, and subsequently stimulate protein synthesis and consequently skeletal muscle growth [359]. SLC36A1 expression was increased following resistance exercise in young and older men [359]. Also, following a single bout of high-intensity resistance exercise, SLC36A1 expression increased amongst many other skeletal muscle amino acid transporters in both healthy men and women, independent of age [360]. SLC36A1 is yet to be characterized in adipose tissue.

TGM2 codes for tissue transglutaminase, a calcium dependent enzyme of the protein-glutamine γ-glutamyltransferases family [361]. It cross-links proteins

between a lysine residue and a glutamine residue, creating an inter- or intramolecular bond that is highly resistant to proteolysis [362]. What proteins get cross-linked and the consequence of that cross-linking in the context of cachexia is yet to be investigated. TGM2 −/− and F13a1−/− double null mice showed no loss of bone mass and maintained normal bone mineral density to 12 months age. Bone marrow adipocity showed large increases in both fat percent (+70.7%) and adipocyte numbers (+65%) suggesting that TG2 and FXIIIA might regulate an osteoblast-adipocyte switch via fibronectin matrix stabilization [363, 364].

TGM2 is downregulated in bone marrow in response to age as it was found to enhance cell growth and survival through anti-apoptosis signaling which is usually downregulated during aging [365]. TGM2 was one of several genes that have higher expression in human brown adipose tissues compared to white adipose tissues [366].

6.2 Downregulated Genes

6.2.1 Results

Table 28: Downregulated Overlapping Genes.

Gene Symbol	Gene Full Name	Muscle		Fat	
		Fold change	P-Value	Fold change	P-Value
AC007246.3	Antisense. Uncharacterized LOC728730	-2.98	0.0166	-2.23	0.019
GPLD1	Glycosylphosphatidylinositol Specific Phospholipase D1	-6.27	0.0172	-2.22	0.012
GSTT1	Glutathione S-Transferase Theta 1	-2.73	0.0108	-3.40	0.046
LOC283731	Uncharacterized LOC283731	-13.65	0.0006	-4.04	0.045
PPP1R9A	Protein Phosphatase 1, Regulatory Subunit 9A	-2.62	0.0052	-2.04	0.022
SYNM	Synemin, Intermediate Filament Protein	-2.26	0.0126	-2.26	0.035

6.2.2 Discussion

GPLD1 codes for phosphatidylinositol-glycan-specific phospholipase D1 enzyme[367]. Many proteins at the extracellular plasma membranes are attached via a glycosylphosphatidylinositol (GPI) anchor. GPLD1 is a GPI degrading enzyme that hydrolyzes the inositol phosphate linkage in proteins anchored by GPI thereby allowing release of the attached protein [368]. In vitro, GPLD1 overexpression is associated with increased expression of *de novo* lipogenesis genes [369]. Also, insulin resistance in human subjects was found to be associated with increased serum levels of GPLD1 which is also involved in lipid metabolism (triglyceride metabolism) [370]. It could be, therefore, a gene that is downregulated in cachectic muscle and adipose tissue as a compensation for the presence of insulin resistance. Despite this evidence, the role of the gene in muscle has not yet been investigated.

GSTT1 codes for glutathione S-transferase theta-1, that is a member of a superfamily of enzymes that catalyze the conjugation of reduced glutathione. Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In healthy states, the majority of glutathione are in the reduced form (GSH). The ratio of oxidized-to-reduced (GSSG-to-GSH) is considered indicative of oxidative stress [371]. GSTT1 functions on the reduced glutathione. GSTT1 is expressed in both, muscle [372] and adipose tissue [373].

Fat deposition was investigated in fat vs. lean chicken. GSTT1 was found to be one of the differentially upregulated genes in adipose tissue of fat chicken [374].

GSTT1 was found to co-vary with NNAT expression in murine fed high fat diet white adipose tissue [373]. NNAT is an acute diet-responsive gene in white adipose tissue and hypothalamus; it may play an important role in metabolism, adipogenesis, and resolution of oxidative stress and inflammation in response to dietary excess [373].

Thus, this decreased expression of GSTT1 in the skeletal muscle and adipose tissues of cachectic subject could reflect reduced cellular ability to manage free radicals and consequently altering cellular functions.

PPP1R9A codes for protein phosphatase 1, regulatory subunit 9A. The PPP1R9A protein is involved in actin cytoskeleton dynamics and in synaptic formation and function [375, 376]. PPP1R9A is also imprinted mainly in skeletal muscle [376]. It is found to be highly concentrated in the synapses of developed neurons [377]. It is suggested that downregulation of PPP1R9A and other genes is an evidence that deficiencies in cytoskeletal dynamics play a role in Huntington's disease pathogenesis [378]. PPP1R9A was one of many expressed genes that were stably retained in H9-hESCs cell line throughout differentiation [379].

SYNM (synemin or desmuslin) codes for an intermediate filament (IF) protein [380]. IFs function by integrating mechanical stress and maintain structural integrity in eukaryotic cells. Diseases involving Ifs have defects in the organization of the contractile apparatus of skeletal and cardiac muscle [381]. SYNM was mainly studied in the sarcomere of skeletal myocytes [380]. It localizes at the Z-disk and has been shown to act as a mechanical linker, transmitting force laterally throughout the tissue, especially between the contractile myofibrils and extracellular matrix [380]. Synemin is expressed mainly in heart and skeletal muscle [380].

Synemin knockout mice have a mild skeletal muscle phenotype, characterized by decreased fiber size and increased sarcolemmal deformability and susceptibility to injury [381]. On the other hand, abnormal accumulations of synemin and desmin in muscle fibres was seen in multiple myopathies [382]. Other study on synemin knockout mice showed a higher hypertrophic capacity with increased maximal force and fatigue resistance following mechanical overload, compared with control mice

[383]. Molecularly, this increased remodeling capacity was accompanied by the decreased expression of myostatin and atrogin expression, and increased follistatin expression [383].

Knockdown of synemin expression with siRNAs within mammalian cells resulted in significantly compromised cell adhesion and cell motility, suggesting a possible role of synemin as a focal adhesion molecule that is essential for cell adhesion and migration [384].

Synemin upregulation or downregulation has marked effects on skeletal muscle. In cachectic patients, the downregulation of expression of this gene may have put the muscle under higher susceptibility for injury while decreasing the fiber size and contributing to the wasting phenotype. However, so far, no data is available about the role of synemin in adipose tissue.

Chapter 7: Overall Discussion

Cancer cachexia is a multifactorial syndrome that is responsible for at least one-third of cancer-related death worldwide and contributes to the death of many others. More than 80% of cancer patients are cachectic towards the end of life. Despite intensive research, the mechanisms of cancer cachexia are still poorly understood and its multi-faceted etiopathogenesis has never been thoroughly reflected on in a single set of experiments. *In vitro* and *in vivo* experimentation, so far, has not led to any leap in our understanding of cachexia, since no model is able to recapitulate the whole spectrum of the cancer cachexia syndrome. In addition, the few conducted clinical studies using microarray had neither given us good insight, nor were reproducible.

It is our believe that identification of early changes in gene expression in cachexia target tissues (muscle and adipose tissue) will lead to an improved understanding of the mechanisms that trigger this important problem in cancer patients. Next generation sequencing is the state-of-the-art technique to generate high throughput, high quality results. This technique has proven to be much more powerful and discriminatory than microarray and is now considered to be the state-of-the-art.

The decisions to include all of the cachectic and non-cachectic cancer patients was made solely based on body weight changes in the past 3-6 months and by meticulous exclusion of samples that might have reflected any etiogenesis that could contribute/aggravate cachexia (i.e. malnutrition, muscular/endocrine/other metabolic diseases, etc.). The cachectic and the weight-stable group showed no significant differences in any of the measured biochemical parameters. The Glasgow prognostic score, which is suggested to be a measure for diagnosing and a guide for treatment of cachexia, failed to distinguish a signature difference in the

cachectic group. It contrast it revealed more advance scores in non-cachectic controls. However, our small sample size cannot fully refute the possibility of that measure to predict cachexia if applied on a larger scale of samples. Yet, we believe it may not be the method of choice for looking at early stages of cachexia.

7.1 Changes in Gene Expression in Skeletal Muscle

Genes whose expression changed in cachectic muscles reflect of a large spectrum of genes encoding skeletal muscle structural proteins, proteins in the muscle contractile machinery, energy metabolism, and others.

Several skeletal muscle proteins in the basement membrane were upregulated, namely agrin, perlecan, collagen, laminin, and neurexin (Figure 8). Agrin is required for the organization of the cytoskeleton and amelioration of function in diseased muscle [385]; perlecan is important in maintaining muscular integrity [386]; collagen and laminin are upregulated during endurance training [387]. On the other hand, many genes encoding transmembrane and intracellular muscular structural proteins were downreguated, such asintegrins, sarcospan, elements of sarcoglycan complex, myotilin, desmin, titin, and α-catinin. Collectively, the functions of the upregulated genes in the basement membrane of myocyte reflect a defensive adaptive protective response to maintain muscular homeostasis which is possibly triggered by the down regulation of essential interacellular proteins.

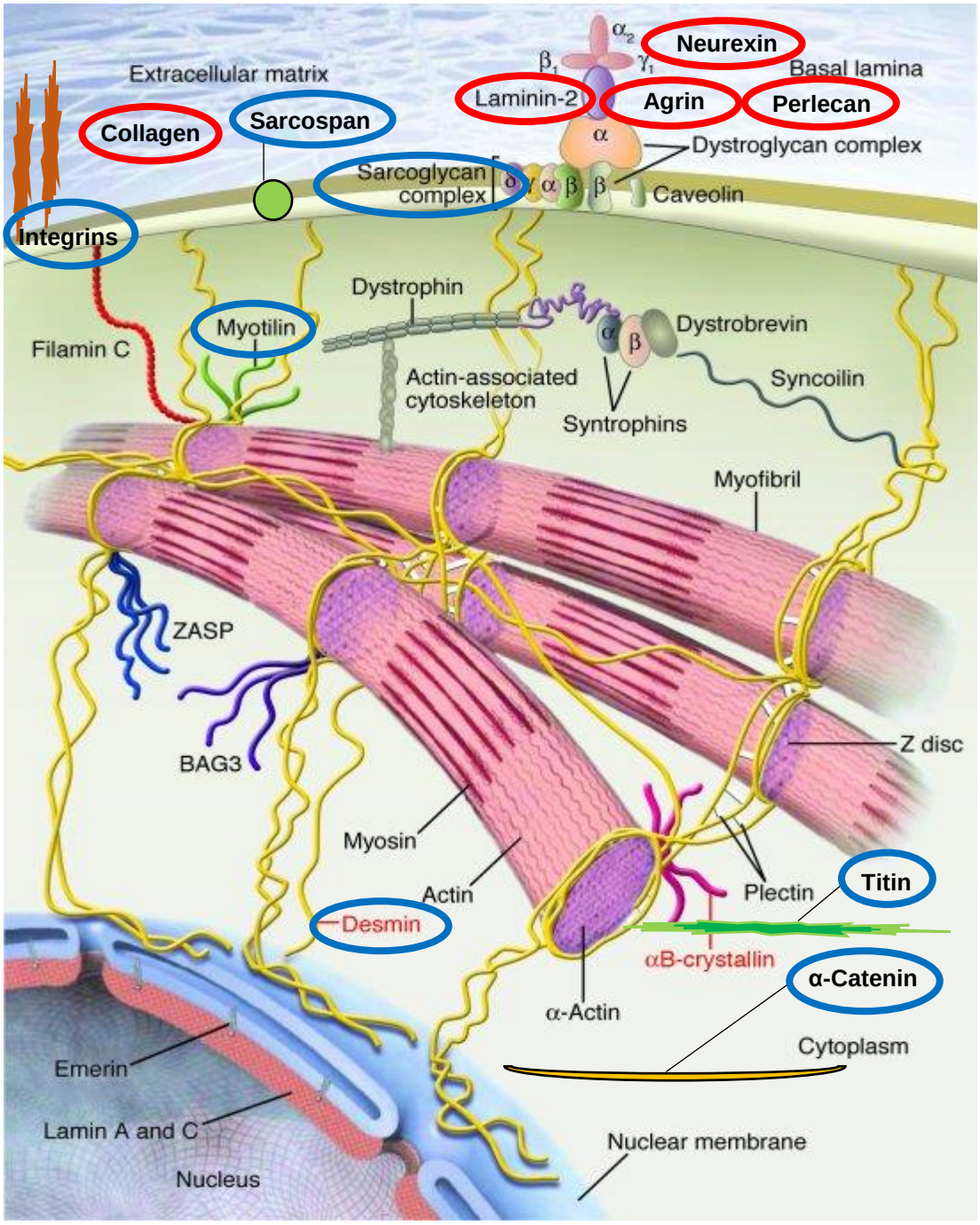

Figure 8: Skeletal Muscle Extracellular Matrix and Structural Muscle Proteins.

Items circled in red are upregulated; items circled in blue are downregulated. Amended from [388].

In addition, integrins, titin [389], and tropomysin [390], which were also downregulated, are essential component in the contractile machinery (Figure 9). Normally, an action potential causes skeletal muscle to depolarize opening the voltage-gated L-type calcium channels (two subunits of which are downreguated). This increases intracellular calcium causing it to bind to calmodulin, which in turn activates myosin light chain kinase (MLCK) (both of which are downregulated). MLCK then phosphorylates the regulatory light chains of the myosin heads leading to a cascade of events that causes muscle contraction (Figure 10). Both, the signaling cascade and the structural response machinery are altered in the muscle of our cachectic patients. Another interesting change in expression was seen in genes involved in the cytoskeleton and myofibers. This indicates that morphological changes can be also evident in cachectic muscles before/during the cachectic changes. Expressions of genes implicated in cellular trafficking were also affected.

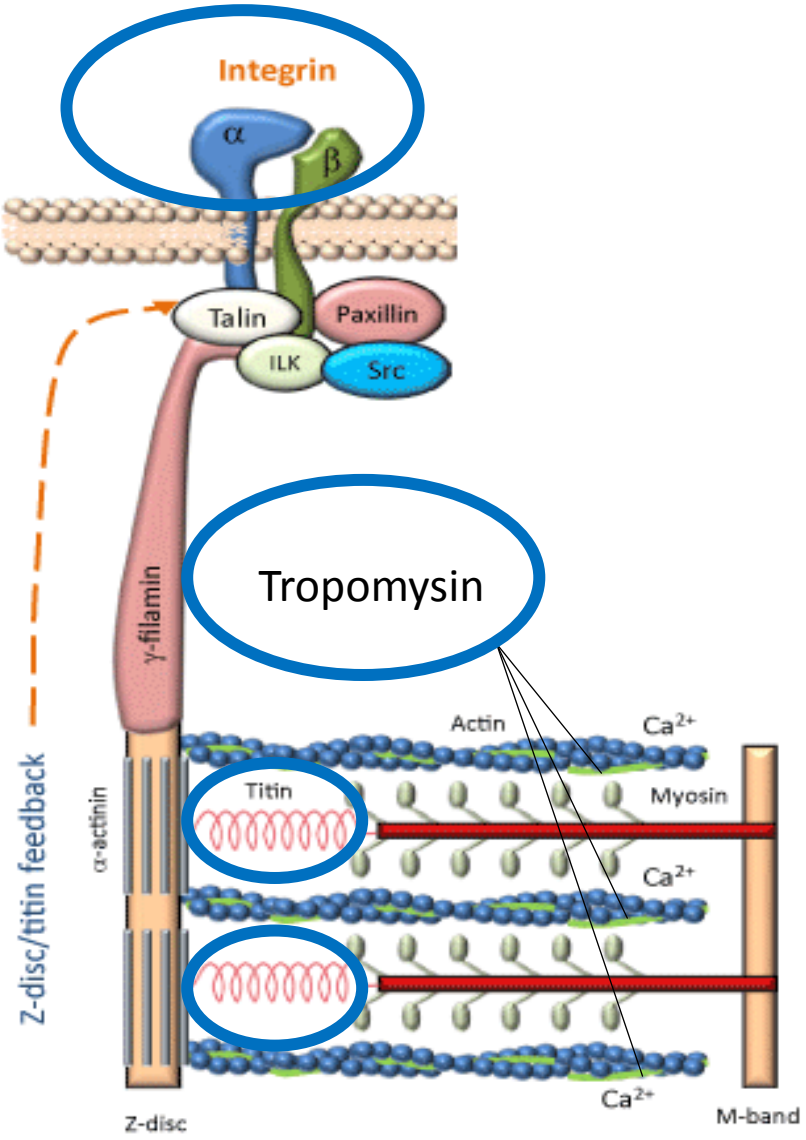

Figure 9: Muscular Contractile Machinery.

Items circled in blue are downregulated. Amended from [391].

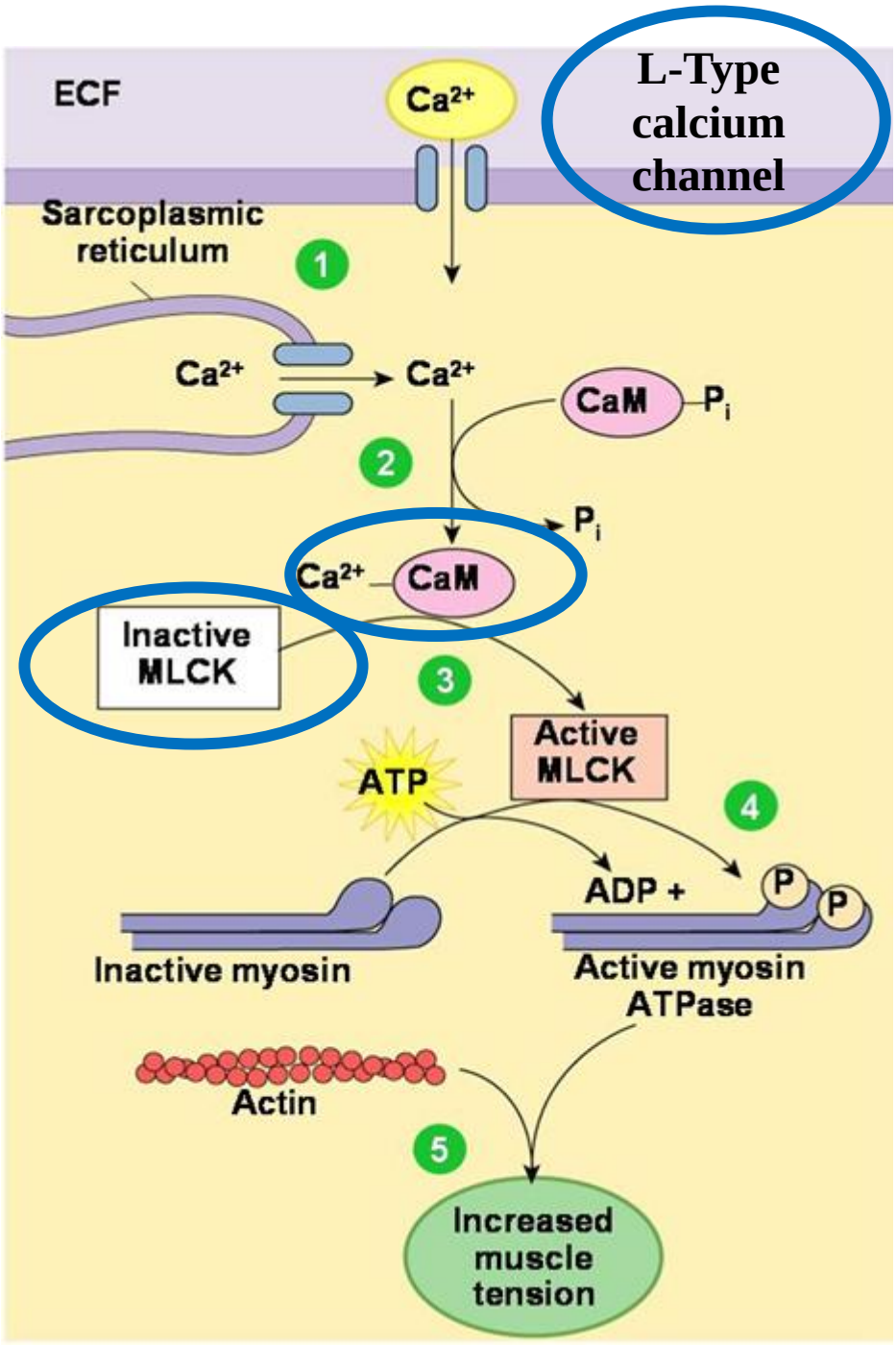

Figure 10: Molecular Signaling in Muscular contraction.

Items circled in blue are downregulated. Amended from [392].

Glycogen is an immediate reserve source of energy for skeletal muscle. Many disorders of glycogen metabolism (as mentioned above) result in muscular phenotypes. Many genes encoding proteins or protein subunits that are necessary components in the machinery needed for glycogen metabolism were downregulated in the cachectic muscles (Figure 11). The involved genes are necessary for either the initiation or progression of glycogenesis (e.g. PGM1; encoding phosphomutase, protein hosphatase; PPP1R3A, PPP1R3B, and PPP1R3C; necessary for activation of glycogen synthase, and AGL; encoding glycogen branching enzyme), or glycogenolysis (Phosphorylase kinase; PHKA1, PHKB, PHKG1; necessary for activation of glycogen phosphorylase) (Figure 11). Athletes experiencing a phenomenon called "hitting the wall", which is caused by depletion of glycogen stores [393], often experience sudden fatigue and loss of energy [394];two symptoms repeatedly reported by cachectic patients. Such alteration in the glycogen turnover in cachectic patients limits energy availability and redirects muscle cells to energy from other sources, such as fat and protein catabolism.

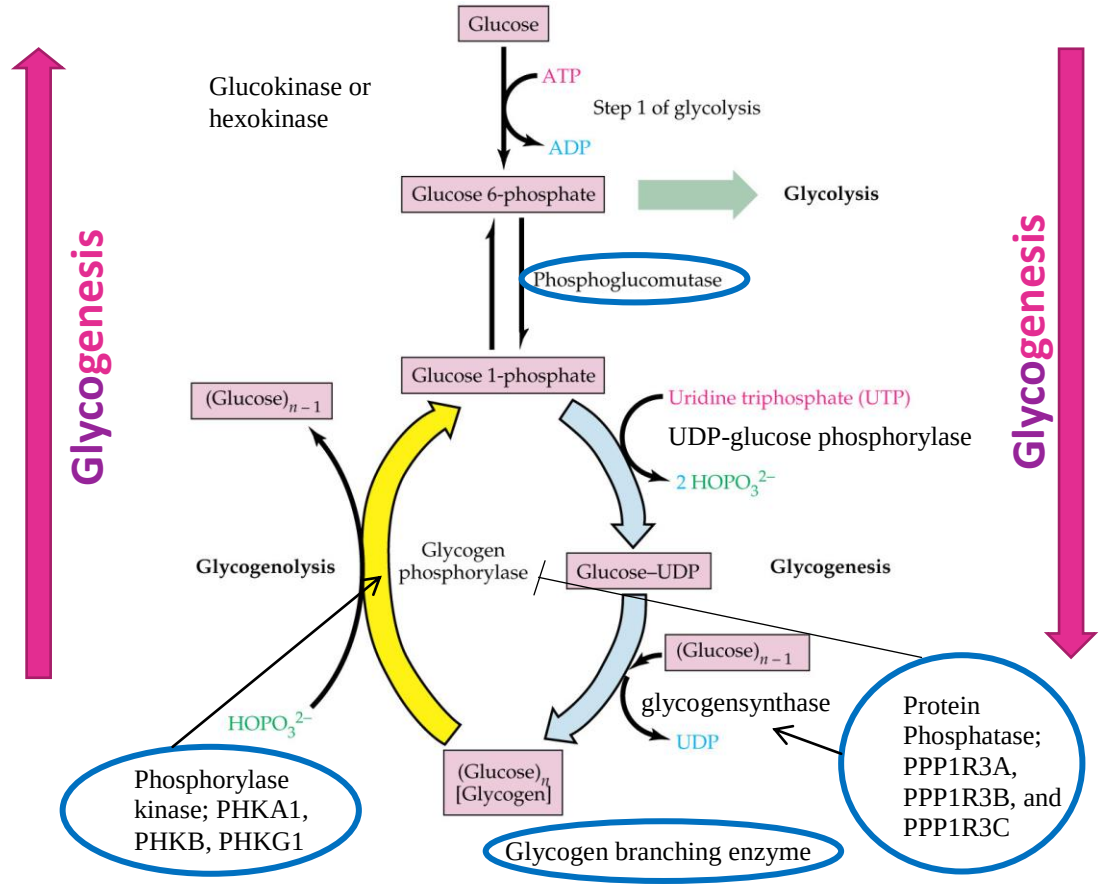

Figure 11: Glycogen Metabolism.

Items circled in blue are downregulated. Amended from [395].

Aerobic cellular respiration is responsible for the most efficient energy production in cells[396]. Interestingly in our findings, the machinery responsible for that process was found to be downregulation at many levels. Normally, aerobic respiration is initiated by glycolysis leading to the production of pyruvate. Pyruvate is then converted into acetyl-CoA via a 3-step sequence of enzymatic activities mediated by "pyruvate dehydrogenase complex". Dihydrolipoyl dehydrogenase (E3) enzyme that is responsible for the last step of the process was found to be downregulated. Acetyl-CoA should then be used in the "citric acid cycle" to generating NADH(nicotinamide adenine dinucleotide) [159]. Three enzymes (dihydrolipoyl succinyltransferase, succinyl-CoA synthetase, and succinate dehydrogenase) in this multistep process were found to be downregulated. NADH can finally enters the oxidative phosphorylation via the electron transport pathway [159], which is itself has several downregulated genes in its 5 different complexes (Complex I: NDUFB5 and NDUFS1; complex II: SDHC; complex III: CYCS and UQCRC2; complex IV: COX20; and complex V: ATP5B).

Our evidence of the disrupted aerobic cellular respiration and the clinical evidence of an increased lactate production in muscle of cachectic patients [397], is suggestive of the possible domination of the anaerobic respiration. This process occurs in animal cells under hypoxic (or partially anaerobic) conditions in oxygen starved muscles. In cachectic patients, hypoxic conditions might not be the direct cause for such switch but rather the slowed aerobic respiration process with increasing demands of muscles for energy production. In many tissues, this is a cellular last resort for energy that most animal tissue cannot tolerate for an extended period of time. It is an energy wasting process that requires lactate to be recycled back into glucose by the liver. The production of lactate via glycolysis as a source of energy and the followed recycling results in a net loss of 4 ATP molecules which could be

the direct cause of cachexia due to an increasing demand of tissue catabolism to meet the required demand of energy (Figure 12).

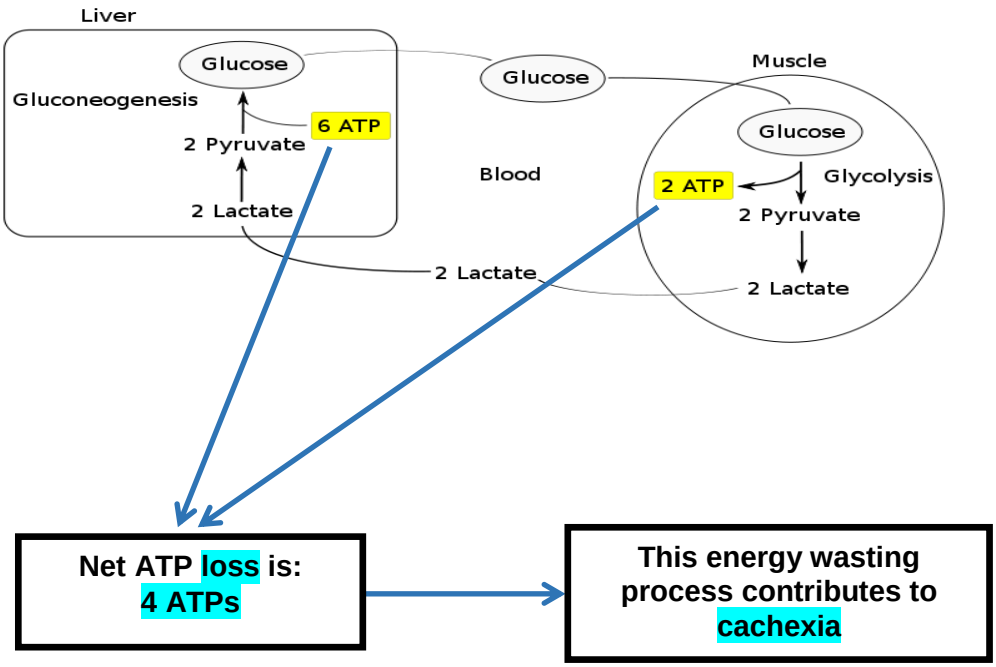

Figure 12: Liver Lactate Recycling and Net ATP Production.

The pentose-phosphate pathway is a parallel pathway to glycolysis [398]. The reaction sequences of central metabolism, glycolysis and the pentose phosphate pathway provide essential precursors for nucleic acids, amino acids and lipids [398]. The upregulation of this pathway (Figure 13) is one finding in this study that reflects adaptation or compensatory muscular changes. We also found upregulation of genes involved in skeletal muscle proliferation, differentiation, and regeneration. We believe that increased expression of these genes reflects a compensatory mechanism that is attempting to maintain muscular homeostasis. These above mechanisms require adequate and strong cell-cell contacts which were evident with the upregulation of many cell adhesion molecules and extracellular matrix pathway genes.

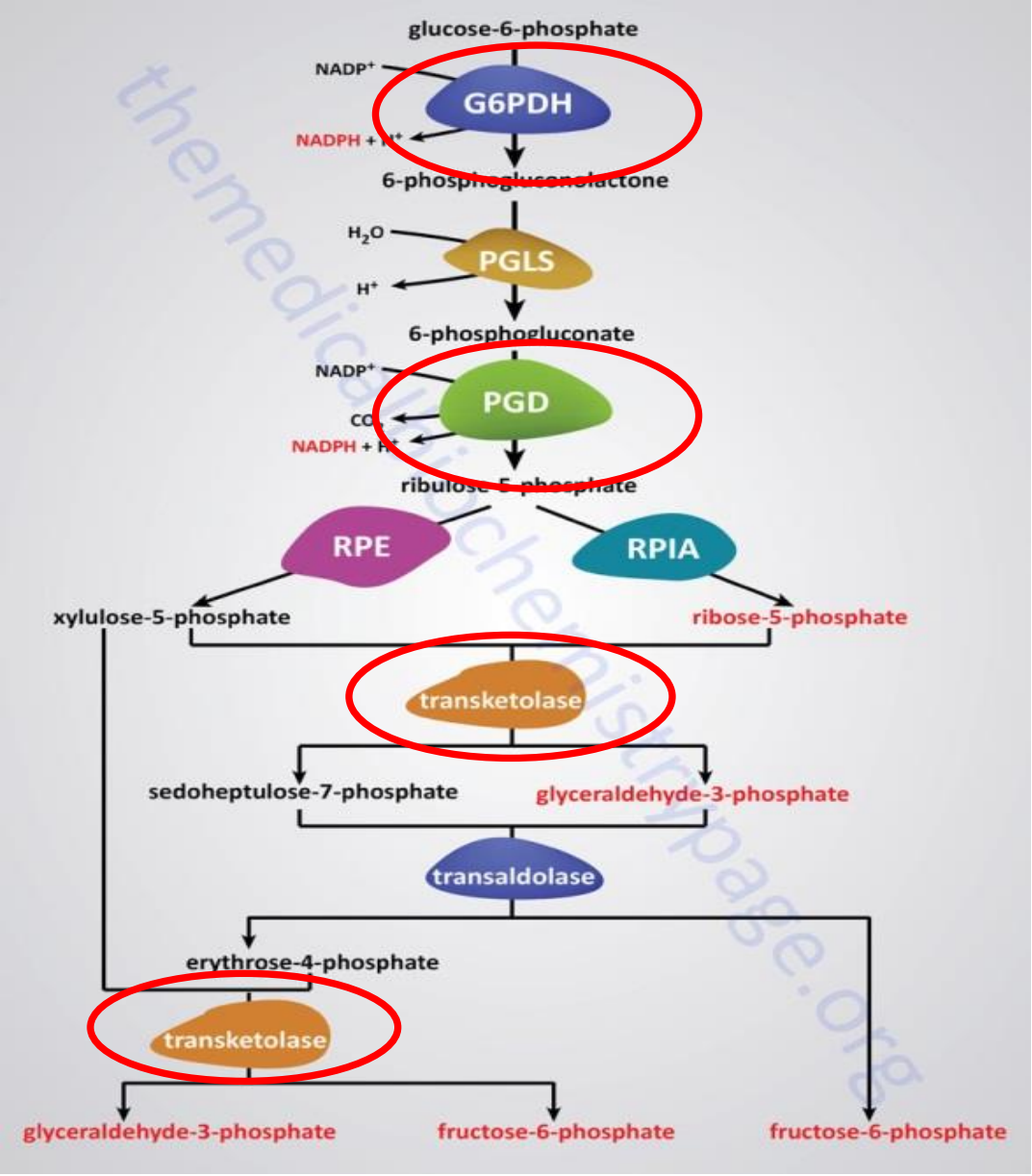

Figure 13: Pentose Phosphate Pathway.

Items circled in red are upregulated. Amended from [399].

Symptoms of cachexia were also reflected in changes in gene expression. Weakness and easily fatigability may also be explained by changes in expression of multiple genes implicated in maintenance of muscular tone, and contractile machinery. In addition, many of the downregulated genes have a documented evidence of genetically inherited muscular ailments which are known to affect muscular tonicity and contractile ability. Also, expression of multiple genes that are implicated in insulin resistance, a key feature of cachexia, were significantly altered.

The major degradation machinery (the ubiquitination pathway) was surprisingly downregulated with reduction in expression of multiple genes and at various different levels. The ubiquitin system has been repeatedly implicated in the preclinical models as the major mechanisms leading to wasting in many *in vitro* and *in vivo* experiments. All clinical studies have failed to reproduce any upregulation of this degradation system in human skeletal muscle biopsies. However, amongst other ground breaking changes in the signature of the cachexia, the present study is the first to show that the pathway is actually significantly downregulated, perhaps in compensation for the wasting triggers. This gave us a reassurance and evidence that our samples represent true early cachectic changes that are subject to bodily defensive compensatory mechanisms attempting to maintain homeostasis.

None of the inflammatory or tumor specific factors were significantly altered. For example, no transcripts for the dermicidin gene, which contains the sequence that codes for the backbone peptide of proteolysis-inducing factor, were detected. Although of course their expression in the tumors themselves may well be altered. Furthermore, the genes encoding inflammatory cytokines and their major receptors were not found to be significantly altered. In contrast, inflammation is a direct cause for cachexia in conditions such as sepsis, severe trauma, or burns. But cancer

cachexia has a slower progression phenotype and may be mediated by inflammatory cytokines, at least in part, in its advanced stages.

The myostatin pathway is at the top of the research agenda for cachexia researchers. Many animal studies strongly correlated its upregulation to the pathogenesis of wasting. However, expression of myostatin in our study was significantly decreased as was its receptor, possibly reflecting end organ adaptation to tumor produced myostatin.

One unique and interesting gene is TIE1. Expression of this gene was significantly increased in a previous human microarray study and was also upregulated in the present study. The TIE1 gene is not well characterized, however, its upregulation in two separate genome-wide expression studies certainly warrants further attention.

7.2 Changes in Gene Expression in Adipose Tissue

Since cachexia is predominantly associated with skeletal muscle changes, changes in adipose tissue have received much less attention with regard to the pathogenesis of cachexia. Many of the genes implicated to be involved from the results of the present study are either uncharacterized or have not been previously studied in adipose tissue. However, several of these encode metabolic enzymes and receptors.

Expression of leptin was downregulated as expected from many previous studies. This is indicative of depleted fat stores and should result in increased feeding (Figure 14). However, as has been discussed the hypothalamus does not respond normally to decreased leptin in the cachectic state. Similar to leptin, tenomodulin downregulation is also reflective of fat depletion [330] and further support the involvement of fat wasting in the cachectic process.

Expression of zinc-α-2-glycoprotein (lipid mobilizing factor) was significantly upregulated as expected. On the other hand, the expression of other genes previously implicated with cachexia, such as hormone sensitive lipase and adipose tissue triglyceride lipase, were unchanged.

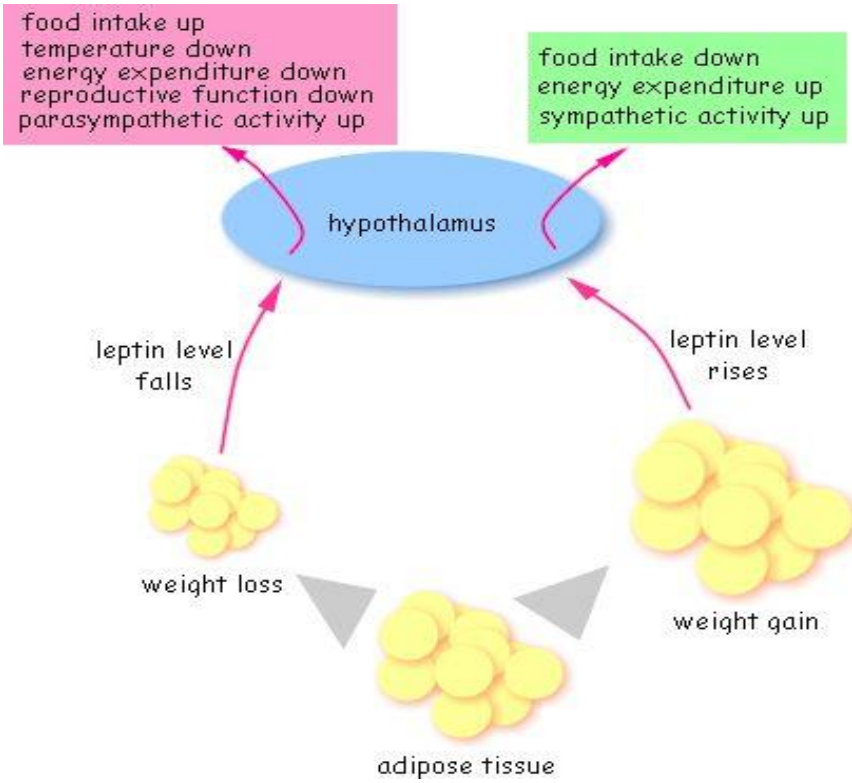

Figure 14: Leptin Production and its Function in the Central Nervous System. Amended from [400].

Expression of genes that are involved in adipogenesis and implicated in insulin resistance were also upregulated. On the other hand, expression of genes that resist lipolytic forces were also upregulated, perhaps in an attempt to neutralize the cachectic state. With the exception of leptin, there were no changes in expression of any genes previously implicated implicated in cachexia, from either clinical or preclinical studies, that were reproduced in our the present study on adipose tissue.

7.3 Changes in Gene Expression in Both; Muscle and Adipose Tissue

With two large organs in the body, there must be a cross-talk mediating the compensation or decompensation signals leading to wasting. Several altered genes were found to be common between these two tissues. Many of these genes are yet to be characterized while others need to be investigated at the context of the pathogenesis of cachexia.

7.4 Conclusion

Progress in cancer cachexia research was, up till now, held back with poor progress due to the lack of reliable and representative sample that serve as a "point of reference" or a benchmark for future research. Cancer cachexia research continuum have either worked in isolated *in vitro* and *in vivo* models which outcomes rarely been a true reflection of what is seen in the clinic. In addition, studies conducted on clinical samples didn't feed enough data reflecting the true complexity of the clinical presentation.

Our enthusiasm took us back to the clinic to extract a meaningful representative sample from cachectic cancer patients exhibiting early cachectic changes. We were able for the first time to reflect on the big picture of the pathological changes in the two major organs affected by cachexia (i.e. muscle and fat). This was achieved by combination of factors that starts by looking at expression changes in early stages of cachexia using simple diagnostic criteria, building on previous array studies where we learnt from their limitations, and finally by utilizing the state-of-the-art technology for generating the highest quality data. These studies confirmed that for a multifactorial condition, genome wide transcriptome analysis is the method of choice to explore the disease complexity. They explain some documented evidence in

cachexia pathogenesis, highlight ambiguous data from animal models, and reveal unexpected changes in gene expression that underlie the pathophysiology of the cachectic state in cancer. These results bring reliable, representable, and consistent data from the clinic and back to the bench with more focused insights to be investigated and verified.

Chapter 8: Limitations and Future Directions

8.1 Limitations

We were lucky that couple of genome-wide array studies have been published during the time we were collecting our samples. We build part of our success by starting at where they have ended. However, no study is limitation free. Due to feasibility issues (time and accrual rate), the study couldn't further restrict its inclusion criteria further (i.e. same age group, gender, diagnoses, etc). This may have in part question the homogeneity of samples. However, we don't believe it is a major influence in the major target organ (i.e. skeletal muscle) as was evident in the muscle heat map. Functional assessment of subjects was not all done. For example, none of the subjects were anorexic or malnourished at the time of obtaining samples. On the other hand, other phenotypes (or symptoms) known to accompany cachexia were not assessed (e.g. weakness and fatigability). Patients were generally with good performance status. Thus, we believe that such symptoms are only clearly evident with advanced cachexia. Our data documented the involvement of many related genes which is a prerequisite to an evident functional impairment.

We were also not able to validate the data for all the differentially expressed genes. It would have been wise if a larger separate group of samples is collected and to get the data cross-validated, however, due to slow accrual rate, we restricted our findings to what in hand.

8.2 Future Directions

Sample collection will continue using the same simple criteria to identify cachectic patients in clinic using meticulous follow-up and recording of weight changes to diagnose patients with cancer cachexia and separate them from non-cachectic

weight-stable controls. After this logical presentation of the complexity of cachexia pathogenesis, the second best step is to be able to verify these changes on a different and larger group of patients. Also, all reproducible changes should be confirmed at protein level using western blotting and immunohistochemistry. Changes in muscular morphology are also worth being investigated via electron microscopy.

In future studies, we would measure circulating factors where changes were seen in the present study, including inflammatory cytokines, GAD15, leptin, mysotatin, proteolysis inducing factor, lipid mobilizing factor, etc.

Finally, it would be hard to completely characterize the role of all of these factors in the development of cachexia unless we succeed in developing a reliable animal model that truly reflects cachexia presentation in humans.

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