Suppression of Skeletal Muscle Turnover in Cancer Cachexia: Evidence from the Transcriptome in Sequential Human Muscle Biopsies

Iain J. Gallagher¹, Nathan A. Stephens¹, Alisdair J. MacDonald¹, Richard J.E. Skipworth¹, Holger Husi¹, Carolyn A. Greig¹, James A. Ross¹, James A. Timmons², and Kenneth C.H. Fearon¹

Abstract

Purpose: The mechanisms underlying muscle wasting in patients with cancer remain poorly understood, and consequently there remains an unmet clinical need for new biomarkers and treatment strategies.

Experimental Design: Microarrays were used to examine the transcriptome in single biopsies from healthy controls (n = 6) and in paired biopsies [pre-resection baseline (weight-loss 7%) and 8 month post-resection follow-up (disease-free/weight-stable for previous 2 months)] from quadriceps muscle of patients with upper gastrointestinal cancer (UGIC; n = 12).

Results: Before surgery, 1,868 genes were regulated compared with follow-up (false discovery rate, 6%). Ontology analysis showed that regulated genes belonged to both anabolic and catabolic biologic processes with overwhelming downregulation in baseline samples. No literature-derived genes from preclinical cancer cachexia models showed higher expression in baseline muscle. Comparison with healthy control muscle (n = 6) revealed that despite differences in the transcriptome at baseline (941 genes regulated), the muscle of patients at follow-up was similar to control muscle (2 genes regulated). Physical activity (step count per day) did not differ between the baseline and follow-up periods (P = 0.9), indicating that gene expression differences reflected the removal of the cancer rather than altered physical activity levels. Comparative gene expression analysis using exercise training signatures supported this interpretation.

Conclusions: Metabolic and protein turnover–related pathways are suppressed in weight-losing patients with UGIC whereas removal of the cancer appears to facilitate a return to a healthy state, independent of changes in the level of physical activity. Clin Cancer Res; 18(10); 2817–27. ©2012 AACR.

Introduction

Cancer cachexia has been defined as “a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass that cannot be fully reversed by conventional nutritional support and that leads to progressive functional impairment” (1). Cachexia impacts negatively on the quality of life of patients with cancer, response to treatment and survival, and thus tackling cachexia should be a central component of patient treatment. Skeletal muscle mass is maintained by a balance between protein synthesis and degradation, and these, in turn, are principally regulated by physiologic inputs such as nutritional status and physical activity. Several systems contribute to intracellular protein breakdown including the ubiquitin/proteasome pathway (UPP), autophagy (lysosomal pathway), caspases, cathepsins, and calcium-dependent calpains. Protein synthesis begins with the association of met-tRNA and the 40S ribosome, a process regulated by elongation and initiation factor 2 (eIF2), which, in turn, is influenced by external signals potentially integrated through the mTOR (2). The balance of activity of these processes represents the molecular basis for muscle mass homeostasis.

Preclinical models of muscle atrophy, which tend to reflect acute and rapid changes in muscle mass, have highlighted the role of the UPP with increased mRNA expression of 2 muscle-specific E3 ubiquitin ligases, MuRF-1/Trim63, and MAFbx/atrogen-1/Fbxo32 (3, 4) and evidence for these enzymes playing a direct role in muscle protein breakdown (5, 6). Transcriptional regulation of these enzymes involves the FOXO1 and FOXO3a transcription factors, which are thought to regulate both the autophagy (7, 8) and the UPP pathways (9). These transcription factors can be inactivated by AKT, a key regulator of protein
Tend to suggest that human cancer cachexia is not simply the predominant alterations in muscle. Here, we have examined the transcriptional profile of human quadriceps muscle in patients before and after potentially curative surgery for upper gastrointestinal cancer. We show that cancer and weight loss lead to a suppression of transcription for genes involved in both anabolism and catabolism, supporting the idea that muscle turnover is suppressed in cancer cachexia. The finding that changes in the transcriptome are reversible with successful removal of the cancer suggests that optimal benefit might be observed if cachexia therapy was provided during or immediately after oncological therapy, rather than being left to a stage when both the cancer and cachexia are refractory to intervention.

**Translational Relevance**

Cachexia affects 50% of all patients with cancer and contributes to 20% of cancer-related deaths. Although there have been many studies in animal models, there are no longitudinal studies in humans to determine the predominant alterations in muscle. Here, we have examined the transcriptional profile of human quadriceps muscle in patients before and after potentially curative surgery for upper gastrointestinal cancer. We show that cancer and weight loss lead to a suppression of transcription for genes involved in both anabolism and catabolism, supporting the idea that muscle turnover is suppressed in cancer cachexia. The finding that changes in the transcriptome are reversible with successful removal of the cancer suggests that optimal benefit might be observed if cachexia therapy was provided during or immediately after oncological therapy, rather than being left to a stage when both the cancer and cachexia are refractory to intervention.

Several functional studies have examined protein synthesis and protein degradation in rodent models of cancer-associated muscle wasting (10–12) and these data imply involvement of both synthesis and degradation pathways in cancer-associated muscle atrophy in animal models. Exploration of the relative contributions of anabolic and catabolic pathways in humans is more limited and most studies include only a small number of patients. Transcriptional activation of UPP components has been found in the skeletal muscle of patients with pancreatic, upper gastrointestinal (UGI), or liver cancer (13–15) and this resulted in increased protein or proteolytic activity *in vitro* (14, 16). It has also been reported that skeletal muscle calpain, atrogin-1, and MuRF1 mRNA remained unchanged in the skeletal muscle of patients with gastric cancer, whereas calpain activity was elevated even in the absence of weight loss (17). In patients with lung cancer, there was no increase in skeletal muscle mRNA expression of UPP genes but cathepsin B mRNA was increased (18).

In contrast to the individual gene observations described above, we found no evidence for an increase in proteolytic gene expression, using genome-wide transcript analysis of 3 types of skeletal muscle, in a relatively large group of weight-losing patients with UGI cancer (19), yet the same approach clearly showed that these systems were activated in human muscle during the more extreme situation found in the intensive care unit (20). The transcriptional analysis was also supported by a lack of alteration in FOXO protein activation; however, protein analysis suggested that CaMKII activation may mediate some aspect of suppressed protein synthesis.

In general observations from cross-sectional studies of cancer cachexia are impacted by patient heterogeneity, including different phases of cachexia. Nonetheless, these tend to suggest that human cancer cachexia is not simply “runaway” proteolysis. One approach to reduce the impact of patient heterogeneity is to study skeletal muscle changes longitudinally. Furthermore, to determine the potential relevance of the tumor burden per se, determination of the skeletal muscle profile before and after removal of the tumor should be insightful. Thus, in the present study, we produced a global transcriptional profile of human quadriceps muscle in patients before and after potentially curative surgery for UGI cancer. We show that the presence of cancer and concurrent weight loss leads to a suppression of transcriptional activity for genes involved in both anabolism and catabolism, supporting the idea that overall muscle turnover is suppressed in cancer cachexia. Critically, we show that the molecular impact of tumor removal on skeletal muscle phenotype appears independent of patient physical activity, suggesting direct cross-talk between the tumor and the process of cancer cachexia.

**Materials and Methods**

**Subjects**

A total of 18 subjects were recruited; 6 healthy control and 12 patients with UGIC. Patients with UGIC had a diagnosis of esophageal (*n* = 6), gastric (*n* = 2), or pancreatic (*n* = 4) malignancy and underwent potentially curative surgery. 8 patients had stage III, 2 patients had stage II, and 2 patients had stage I disease. Five patients had completed a course of neoadjuvant chemotherapy but had not received chemotherapy in the 4 weeks before surgery. No subjects were taking anabolic/catabolic agents and had uncontrolled diabetes or known thyroid disorders. The weight-stable healthy control comprised 5 subjects undergoing abdominal surgery for nonmalignant, noninflammatory conditions and 1 subject recruited from the community. Written informed consent was obtained from all subjects and ethical approval received from Lothian Research Ethics Committee (Scotland, UK). Body weight was measured with participants in light clothing using a beam scale (Seca). Height was measured using a standard wall mounted measure and body mass index (BMI) was calculated. Clinical details and degree of weight loss from self-reported preillness stable weight were recorded.

**Muscle biopsies**

All patients with UGIC and 5 healthy controls underwent a baseline percutaneous quadriceps muscle biopsy following an overnight fast at the time of induction of general anesthesia using conchotoine biopsy forceps. Follow-up biopsies for the patients with UGIC and for the healthy control recruited from the community were obtained following an overnight fast under local anesthesia using a Bergstrom needle with suction. Tissue samples were quickly cleaned of blood, flash-frozen in liquid nitrogen, and stored at −80°C until further analysis.

**Blood measures**

All blood samples were taken following an overnight fast. Albumin and C-reactive protein (CRP) were measured in Clinical Chemistry, Royal Infirmary, Edinburgh (fully...
accrued by Clinical Pathology Accreditation Ltd.) using standard automated methods. A CRP ≥10 mg/L was considered consistent with the presence of systemic inflammation.

**Quadriceps strength**

Maximum voluntary isometric quadriceps strength was measured using an established method (21). The participant was seated in an adjustable straight-backed chair with the pelvis secured and the knee flexed at 90 degrees. A cuff was placed around the ankle and attached via an inextensible chain to a strain gauge and data acquisition system (Powerlab, AD Instruments). Following instruction, the participant made a maximum voluntary contraction which was held for 5 seconds. Three separate measurements were obtained for each limb and the highest value from the dominant limb used in subsequent analysis. Data were normalized to body mass (N kg⁻¹).

**Physical activity monitoring**

Physical activity and sedentary behavior were recorded continuously over a 4-day period using an activPAL activity monitor (PAL Technologies Ltd.) as described previously (22). The monitor was worn at baseline in the week preceding surgery and then in the week following the follow-up assessment. The average number of steps per 24 hours over the 4-day period was calculated and used for analysis.

**Molecular biology**

Tissue was homogenized in QIAzol (Qiagen) reagent using a Polytron PT1200E (Kinematica AG). Total RNA was extracted using miRNeasy columns (Qiagen) as directed by the manufacturer with an on column DNase digestion step. RNA was quantified using the Nanodrop Instrument (Labtech Intl.). Quality and purity of RNA were examined using 260/280 and 260/230 ratios. The Agilent Bioanalyzer (Agilent) was used to assess RNA integrity using previously published protocols (19).

**Reverse transcription quantitative PCR**

The High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used to convert 500 ng of RNA to cDNA following the manufacturer's directions. Quantitative PCR (qPCR) reactions were made up to 10 μL using 5 μL POWER SYBR-Green Master mix (Applied Biosystems), 0.2 μL forward primer, 0.2 μL reverse primer, 3.6 μL H₂O, and 1 μL cDNA. The final primer concentration was 50 nmol/L of each primer per reaction. Reactions were run in triplicate on a StepOne Plus instrument (Applied Biosystems). Running conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Primer efficiencies were examined with reference to β-actin (which did not vary with condition—data not shown). For genes with primer efficiencies comparable with β-actin, we used the ΔΔCt method for analysis. Data from primers with efficiencies that differed from the β-actin primers were analyzed using the standard curve method. Primer sequences are detailed in the Supplementary Material.

**Microarrays**

Reverse transcription of RNA was carried out using the Ovation RNA Amplification System v2 (Nugen Technologies Inc.). One hundred nanograms of total RNA was reverse-transcribed as per manufacturer’s protocol. cDNA was purified using QiAquick PCR purification kit (Qiagen) and quantified using the Nanodrop instrument. For the microarray study, 3.75 μg of cDNA was fragmented and labeled with the FL-Ovation cDNA Biotin Module V2 (Nugen Technologies Inc.) and hybridized to each Affymetrix U133+2 array (Affymetrix) using protocols provided by Nugen Technologies Inc. Arrays were washed, labeled, and scanned following Affymetrix standard procedures. Raw array data were normalized using the MAS 5.0 algorithm to a global scaling intensity of 100. Quality assurance of array data examined the standard quality assessments including scaling factors, glyceraldehyde–3–phosphate dehydrogenase (GAPDH) and β-actin 5′/3′ ratios, RLE and NISE plots, and clustering of raw array data. Although we found consistently high β-actin 3′ to 5′ ratios, an observation we have made previously using the Nugen amplification protocols on muscle-derived RNA (unpublished observation), there were no other indications that any array failed these standard quality assurance procedures. Raw microarray data have been deposited with Gene Expression Omnibus (GEO) under accession number GSE34111.

**Statistics**

Group data are presented as mean and SEM. Comparisons were by ANOVA or Student t test as implemented in R (www.r-project.org) or SPSS (IBM). For multiple testing of reverse transcription qPCR (RT-qPCR) data, P values were corrected using the Bonferroni method. The raw array data were normalized using the robust multivariate analysis (RMA) algorithm. Low variance probes were removed from the data set before assessment of differential regulation. Microarray data were examined using the significance analysis of microarrays (SAM; ref. 23) approach and a false discovery rate (FDR, expected proportion of false-positives) of 10% was applied to select differentially expressed genes. Gene Ontology (GO) and Kyoto Encyclopedia and Genes and Genomes (KEGG) enrichment in differentially expressed genes were examined using hypergeometric t test as implemented in the GOSTats package in R/Bioconductor. A tissue-enriched gene set derived from the array data was used as the background for this analysis to compensate for tissue-specific GO bias (24). We also used the web-based Ingenuity pathway analysis (IPA) to visualize the gene associations modulated in the muscle tissue. IPA relies on bibliometric associations that can provide either insight into the canonical pathways responsible for a pattern of gene changes or simply a visual association network showing how the regulated genes may be interacting, assuming that all components are expressed in the target organ.

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Suppressed Turnover of Skeletal Muscle in Cancer Cachexia

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Results

Demographics

Demographic details for the groups are shown in Table 1. Age was not significantly different between patients with UGIC and healthy controls. BMI and weight in the cancer group did not differ significantly from the control group at any time point. The median time between baseline and follow-up biopsies was 8 months (range, 5–12 months). All patients with UGIC were clinically cancer-free at the time of repeat biopsy. Mean weight loss from self-reported usual weight was 7.4% at baseline and increased to 13.8% at follow-up. However, this did not occur in a linear fashion but rather was more rapid immediately following surgery before stabilizing in the 2 months before follow-up biopsy (Fig. 1A). Eight of the 12 patients with UGIC were included in the activity meter study and neither age and BMI nor weight differed significantly from the overall UGIC group (data not shown). In patients with UGIC, there was an increase in quadriceps strength between baseline and follow-up of 15%, but this did not reach statistical significance ($P = 0.2$; Table 1; Fig. 1C). Overall physical activity (step count) remained stable between baseline and follow-up (Table 1; Fig. 1B).

Albumin and CRP

At baseline, patients with UGIC had reduced albumin levels compared with healthy controls [36.0 (0.5) vs. 42.4 (1.3) g/L, $P < 0.001$; Table 1]. At the time of follow-up biopsy, albumin had returned to within the reference range (36–44 g/L; Table 1). CRP did not differ between baseline and follow-up (9.75 vs. 6.0 mg/L, $P = 0.5$) and was not significantly different compared with healthy controls at either time point (Table 1).

Paired muscle biopsy transcriptome

Using paired samples from the patients with UGIC ($n = 12$) obtained immediately preoperatively (baseline: cancer present; weight-losing) and at a median of 8 months postoperatively (follow-up: cancer resected; weight-stable for at least 2 months), we examined global gene expression using microarrays. Paired analysis within the SAM method (23) identified 1,868 regulated genes with an FDR of 6% and a fold change of at least 30%, 1,747 downregulated and 121 upregulated in baseline samples compared with follow-up samples (Fig. 2A). We validated expression of selected regulated genes by RT-qPCR and found good agreement between the array and RT-qPCR data (Table 2).

To examine the biologic context of the regulated genes, we used the hypergeometric $t$ test to identify enriched Gene Ontology Biological Processes (GOBP) and KEGG pathways. Using a background list of genes detectably expressed in muscle tissue (24), we identified cellular metabolic process as the most enriched biologic process ($P < 0.001$). The enriched GOBP categories included several broad metabolic processes (e.g., metabolic process, primary metabolic process), muscle growth categories (e.g., striated muscle hypertrophy, regulation of muscle hypertrophy), catabolism categories (e.g., endoplasmic reticulum–associated protein catabolic process, ubiquitin-dependent protein catabolic process), and anabolism categories (e.g., response to insulin stimulus, response to protein stimulus). In each of these categories, the genes involved were overwhelmingly downregulated in the baseline samples. Selected categories are presented in Table 3 and full enrichment results are available in the Supplementary Material. Enriched KEGG pathways included the calcium signaling pathway, the peroxisome pathway, and EGF receptor signaling (ErbB signaling pathway).

Table 1. Demographic data for paired samples (baseline and follow-up) and healthy control subjects in the study

<table>
<thead>
<tr>
<th>Paired samples</th>
<th>Baseline ($n = 12$)</th>
<th>Follow-up 2 ($n = 12$)</th>
<th>Controls ($n = 6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender (M/F)</strong></td>
<td>10/2</td>
<td>10/2</td>
<td>4/2</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>65</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>26.4 (1.3)</td>
<td>24.6 (1.3)$^a$</td>
<td>28.2 (1.7)</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>78.2 (4.5)</td>
<td>72.8 (4.4)</td>
<td>84.9 (7.2)</td>
</tr>
<tr>
<td><strong>Change from usual weight (%)</strong></td>
<td>$-7.3 (2.7)^b$</td>
<td>$-13.8 (2.7)^{a,b}$</td>
<td>0</td>
</tr>
<tr>
<td><strong>CRP, mg/L</strong></td>
<td>9.8 (3.6)</td>
<td>6.0 (3.4)</td>
<td>1.8 (1.0)</td>
</tr>
<tr>
<td><strong>Albumin, g/L</strong></td>
<td>36 (0.5)$^a$</td>
<td>42 (0.9)$^a$</td>
<td>42 (1.3)</td>
</tr>
<tr>
<td><strong>Step count/24 h ($n = 8$ per group)</strong></td>
<td>5,607 (1,210)</td>
<td>5,675 (917)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Quadriceps strength, N kg$^{-1}$</strong></td>
<td>3.5 (0.3)</td>
<td>4.5 (0.9)</td>
<td>NA</td>
</tr>
</tbody>
</table>

NOTE: Data (except gender split and age) are presented as mean (SEM). Abbreviation: F, female; M, male; N, Newton.

$^a$Significantly different from baseline ($P < 0.01$, paired t test).

$^b$Significantly different from the controls ($P < 0.01$).
We used IPA to examine a subset of 350 robustly regulated genes that did not overlap with either endurance (25) or resistance training (26) data sets. Network analysis, relying on the IPA database revealed the proteasome, NF-kB, and caspases as potential network hubs connected to many downregulated transcripts (Fig. 2B). Downregulation of these network genes suggests that activation of the hub genes is lower preoperatively supporting the lack of activation of proteolytic genes in the differential expression analysis. Using gene set enrichment analysis and the online oPOSSUM tool, we examined the gene expression data for genes under the control of specific transcription factors and found no significant results, suggesting that no single canonical signaling pathway is driving the muscle cachexia profile.

Preclinical models (3, 4, 27) have identified a set of genes, commonly termed atrogenes, which are involved in cancer-driven muscle wasting. In particular, the levels of the E3 ligases atrogin-1 (FBXO32) and MuRF1 (TRIM63) are elevated in the muscle of cachectic animals. These genes were not differentially expressed in the microarray data, nor when examined by RT-qPCR (atrogin-1 \( P = 1 \); MuRF1 \( P = 0.69 \); paired \( t \) test; Table 4). Examination of the autophagy genes BNIP3 and GABARAPL1 (28, 29) also revealed no changes in expression of these genes between baseline, follow-up, or healthy control groups (Table 4). Glucocorticoids play a role in muscle wasting in some conditions (30). We found no differences in the mRNA levels of the glucocorticoid receptor (NR3C1) and 11\( \beta \)-HSD between the groups (Table 4).

Physical activity
To control for potential changes in physical activity in the baseline versus follow-up periods, the average step count per 24 hours was measured (\( n = 8 \) of 12 patients with UGIC). Mean (SEM) step count/24 hours at baseline was 5,607 (1,210) and 5,675 (917) at follow-up. This difference was not significant (\( P = 0.9 \); paired \( t \) test; Fig. 1B). As a complementary approach to direct measurement of physical activity, we also compared our list of differentially expressed genes with those identified recently in the response to 2 types of exercise training (25, 26). Of the 848 endurance training responsive genes, only 60 overlapped with those regulated between the baseline and follow-up time points (see Supplementary Material) and even fewer overlapped with strength training. To compare the response in cancer cachexia with that seen in dietary restriction, we examined the overlap with the 2,839 differentially expressed genes identified in response to simple dieting (31). Only 12 genes overlapped between our data set and those regulated in skeletal muscle in response to simple dieting. To examine any relationship with the ageing process, we compared our data set with a large study of young versus elderly muscle (manuscript submitted) and found no association between the data sets. Thus, using multiple array analysis strategies we can conclude that the major phenotype of skeletal muscle “responding” to removal of a tumor does not appear related to changes in physical activity, nutritional status, or age.
Comparison with healthy control muscle

Comparison of the muscle from the patients with UGIC at both the baseline and follow-up time points with muscle from weight-stable healthy control subjects revealed 941 genes with lower expression (FDR < 10%) in baseline UGIC muscle compared with healthy control muscle. At UGIC baseline, no genes were expressed at higher levels than in control muscle with FDR < 10%. Of the 941 downregulated genes, 558 were also regulated between the baseline and follow-up samples and all except one (TFDP2, transcription factor Dp-2) were higher at follow-up. Highly enriched GOBP categories included lipid oxidation, catabolic process, protein polyubiquitination, muscle structure development, and striated muscle hypertrophy. Enriched KEGG pathways included fatty acid metabolism, metabolic pathways, peroxisome, oxidative phosphorylation, ubiquitin-mediated proteolysis, and regulation of autophagy (see Supplementary Material). Comparison of gene expression between healthy control muscle and the follow-up samples revealed only 2 genes differentially expressed within the FDR cutoff point (NR1D2 and HDAC9). Thus, the follow-up samples had a transcriptomic profile indistinguishable from healthy muscle, albeit that the total sample size was limited for this unpaired analysis.

Discussion

Cachexia is an important cancer co-morbidity impacting on quality of life and response to treatment. In this study, we examined the transcriptomic response of skeletal muscle before and after potentially curative surgery for UGIC. Resection of UGIC is routinely associated with a weight loss of 7% to 15% of body weight (32) and patients will generally not regain nor return to their preoperative weight. Weight loss during the postsurgical phase is considered to be largely due to loss of fat mass with only limited changes in muscle mass (32), suggesting that greater emphasis should be placed on the restoration of muscle phenotype and function rather than body weight. The present study investigated patients with UGIC undergoing tumor
resection with an average weight loss of 7% body weight (i.e., fulfilling the diagnostic criteria for cancer cachexia; ref. 1). Patients underwent a preoperative baseline quadriceps biopsy with a repeat quadriceps biopsy conducted approximately 8 months later when all patients were clinically disease-free and had been weight-stable for at least 2 months (Table 1; Fig. 1A). There is a marked decline in physical activity immediately following major abdominal surgery. However, by 8 months after surgery, patients’ quadriceps strength tended to be higher than baseline and overall physical activity had returned to baseline levels. It is thus reasonable to consider that the patients in the present study had recovered from the net catabolic effects of both their cancer cachexia and surgery. Sequential paired biopsies allowed analysis of changes in skeletal muscle phenotype during recovery whereas comparison with a group of weight-stable healthy controls allowed determination of the completeness of recovery. In the present study, patients with UGIC showed normalization of muscle phenotype postsurgery (Table 1; Fig. 2A). Strikingly neither the cancer cachexia signature nor the recovery of healthy muscle phenotype appeared to be related to levels of physical activity.

The present study shows that in human skeletal muscle, of the 1,868 regulated genes associated with cancer and weight loss, the vast majority (94%) are downregulated. Category analysis of the differentially expressed genes showed that both anabolic (e.g., muscle organ development, response to insulin stimulus) and catabolic (e.g., ubiquitin-dependent protein catabolic process, regulation of proteasomal protein catabolic process) process gene

### Table 2. RT-qPCR validation of microarray data

<table>
<thead>
<tr>
<th>Gene</th>
<th>Baseline vs. follow-up</th>
<th>Healthy vs. baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Array (FDR)</td>
<td>qPCR (P)</td>
</tr>
<tr>
<td>COMP</td>
<td>3.91 (4.1)</td>
<td>4.3 (&lt;0.01)</td>
</tr>
<tr>
<td>ADIPOQ</td>
<td>3.3 (4.1)</td>
<td>3.2 (&lt;0.01)</td>
</tr>
<tr>
<td>MMP3</td>
<td>3.2 (6.1)</td>
<td>2.7 (0.04)</td>
</tr>
<tr>
<td>PKC1</td>
<td>3.0 (6.1)</td>
<td>2.5 (0.01)</td>
</tr>
<tr>
<td>ANGPTL7</td>
<td>2.8 (6.1)</td>
<td>2.5 (&lt;0.01)</td>
</tr>
<tr>
<td>HSP90AB1</td>
<td>0.45 (6.1)</td>
<td>0.5 (0.04)</td>
</tr>
<tr>
<td>SL25A37</td>
<td>0.45 (6.1)</td>
<td>0.5 (&lt;0.01)</td>
</tr>
<tr>
<td>PROX1</td>
<td>0.45 (2.1)</td>
<td>0.5 (&lt;0.01)</td>
</tr>
<tr>
<td>RCAN1</td>
<td>0.44 (0.0)</td>
<td>0.5 (&lt;0.01)</td>
</tr>
<tr>
<td>HINT3</td>
<td>0.25 (0.0)</td>
<td>0.2 (&lt;0.01)</td>
</tr>
</tbody>
</table>

**NOTE:** Array data are presented as fold change (FDR expressed as a percentage) and RT-qPCR data as fold change (Bonferroni corrected P value for difference in means). N = 12 paired samples for follow-up versus baseline analysis; N = 5 healthy control (for one sample there was insufficient RNA for RT) and 12 baseline samples for healthy versus baseline analysis. RT-qPCR data were analyzed by paired t tests for follow-up versus baseline analysis and by unpaired t tests for healthy control versus baseline analysis.

### Table 3. GOBP categories regulated between baseline and follow-up time points

<table>
<thead>
<tr>
<th>GOBP ID</th>
<th>P</th>
<th>Expected count</th>
<th>Actual count</th>
<th>Annotated genes on array</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0044237</td>
<td>0.00</td>
<td>784</td>
<td>859</td>
<td>5,042</td>
<td>Cellular metabolic process</td>
</tr>
<tr>
<td>GO:006350</td>
<td>0.01</td>
<td>288</td>
<td>320</td>
<td>1,854</td>
<td>Transcription</td>
</tr>
<tr>
<td>GO:0045449</td>
<td>0.00</td>
<td>277</td>
<td>315</td>
<td>1,784</td>
<td>Regulation of transcription</td>
</tr>
<tr>
<td>GO:0009056</td>
<td>0.03</td>
<td>133</td>
<td>153</td>
<td>858</td>
<td>Catabolic process</td>
</tr>
<tr>
<td>GO:0030163</td>
<td>0.01</td>
<td>45</td>
<td>59</td>
<td>288</td>
<td>Protein catabolic process</td>
</tr>
<tr>
<td>GO:0006511</td>
<td>0.01</td>
<td>34</td>
<td>48</td>
<td>216</td>
<td>Ubiquitin-dependent protein catabolic process</td>
</tr>
<tr>
<td>GO:0032868</td>
<td>0.02</td>
<td>15</td>
<td>24</td>
<td>99</td>
<td>Response to insulin stimulus</td>
</tr>
<tr>
<td>GO:0051789</td>
<td>0.03</td>
<td>13</td>
<td>20</td>
<td>85</td>
<td>Response to protein stimulus</td>
</tr>
<tr>
<td>GO:061061</td>
<td>0.04</td>
<td>37</td>
<td>47</td>
<td>236</td>
<td>Muscle structure development</td>
</tr>
<tr>
<td>GO:0007517</td>
<td>0.01</td>
<td>31</td>
<td>43</td>
<td>197</td>
<td>Muscle organ development</td>
</tr>
<tr>
<td>GO:0060537</td>
<td>0.04</td>
<td>20</td>
<td>28</td>
<td>131</td>
<td>Muscle tissue development</td>
</tr>
<tr>
<td>GO:0014706</td>
<td>0.02</td>
<td>19</td>
<td>28</td>
<td>124</td>
<td>Striated muscle tissue development</td>
</tr>
</tbody>
</table>
expression was suppressed. Furthermore, there was a lack of substantive overlap with transcriptomic signatures from endurance training, strength training, ageing, or simple dieting (25, 26, 31, 33). This is perhaps not surprising as each of these situations may be considered within the range of normal physiology rather than pathologic states. We were able to identify the inflammatory transcription factor NF-kB as a hub gene using IPA, however, this gene is linked to many aspects of muscle remodeling including responses to endurance training (25). Other approaches to identify transcription factors [a priori gene set enrichment analysis (GSEA) and post hoc analysis with the web-based oPOSSUM software] did not yield any significant results, suggesting that no single transcription factor network is involved in human cancer cachexia. Limited numbers, variable human data, and transcription factor redundancy are factors that might constrain the discovery of such underlying control genes.

Comparison of the patients with UGIC with weight-stable noncancer healthy controls shows that following removal of cancer and stabilization of weight loss, the transcriptome essentially returns to normal. Taken together, we would hypothesize that muscle turnover is suppressed in the early stages of cancer cachexia, but with successful cancer treatment, these changes are reversible and may directly relate to the presence of the tumor. Our observations may also have implications for the timing of interventions such as physical therapy, as it may be more productive to intervene during this stable period when the molecular phenotype is apparently healthy and function improving. Alternatively, the recovery of the molecular phenotype could be accelerated by earlier interventions and future studies may be able to address this point.

In a recent study on the response of adipose tissue to cancer cachexia, 364 genes were downregulated and 61 genes upregulated in abdominal subcutaneous white adipose tissue (34). Similar to the present study, pathway analysis indicated that downregulated genes were involved in cytoskeleton and extracellular matrix processes. Changes in gene expression were reciprocal to those observed in obesity, suggesting that regulation of fat mass in cachexia may be dominated by the effects of reduced food intake. In the present study, the gene changes in muscle were dissimilar to those seen in simple dieting (31), thereby confirming the essential phenotype of cachexia that the response of muscle to cancer is distinct from simple starvation (1, 35) and cannot be explained by cancer-induced anorexia alone. This observation may also explain the suboptimal skeletal muscle response to nutritional supplementation observed in patients with cancer.

The transcriptomic signal that we detected at baseline in patients with UGIC suggests depression of muscle turnover in patients with cancer-associated weight loss supporting earlier ideas that both global protein synthesis and breakdown were suppressed. While muscle turnover could be a function of changes in physical activity, we did not observe any differences in 24-hour step count between baseline and follow-up assessments and found a biologically relevant improvement of 15% in normalized quadriceps strength after tumor removal. Furthermore, the genes differentially expressed after removal of the tumor showed little overlap with genes expressed after exercise training, supporting the conclusion that restoration of muscle phenotype was not simply a retraining response. The median time interval between operation and follow-up biopsy was 8 months and muscle function is generally considered to be recovered by this time (36).

The loss of skeletal muscle protein in cancer cachexia has been attributed to both alterations in the transcription of specific genes leading to changes in muscle turnover and/or a global reduction in cellular RNA reducing overall translational capacity. A global reduction in RNA abundance might be due to reduced transcriptional capacity as a result of loss of myonuclei secondary to enhanced apoptosis or reduced satellite cell recruitment. We have previously observed evidence of apoptosis in cachectic patients with cancer similar to those involved in the present study (37). However, Lundholm and colleagues reported that human skeletal muscle RNA content is unaffected by the presence of a tumor, whereas in animal models, there was a decrease in RNA content (38). While animal models carrying a variety of tumors have shown reduced RNA content in muscle (39–41) in 2 of these models, there was also evidence for concurrent increases in mRNA expression of proteasomal subunits (15, 40). Notably, we have found a reduction in mRNA expression for proteasomal pathway components in

<table>
<thead>
<tr>
<th>Table 4. RT-qPCR data for selected atrophy-associated genes</th>
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<tbody>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>TRIM63 (MuRF1)</td>
</tr>
<tr>
<td>FBXO32 (Atrogin-1)</td>
</tr>
<tr>
<td>BNP3</td>
</tr>
<tr>
<td>GABARAPL1</td>
</tr>
<tr>
<td>NR3C1</td>
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<tr>
<td>HSD11β1</td>
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NOTE: Data are shown as fold change (Bonferroni corrected \( P \) value for difference between means). Baseline versus follow-up data were analyzed using paired \( t \) tests.
human UGIC in this study and no evidence for changes in mRNA for these components previously (19).

Direct evidence for the involvement of specific proteolytic and synthetic pathways in human cancer cachexia is limited. However, the balance of evidence, based on stable isotope and global transcript analysis, including the present study, appears to favor the conclusion that suppression of both processes occurs, with the reduction in synthetic pathways exceeding suppression of the proteolytic pathways. In line with this, protein degradation as measured by net release of 3-methylhistidine across the leg was lower in patients with cancer than in healthy controls or acutely ill patients and accompanied by anabolic blunting in response to feeding, suggesting a net catabolic status rather than increased degradation (42). Others have also reported depressed skeletal muscle protein synthesis in the face of maintenance of protein breakdown, again suggesting relative catabolic advantage (43).

What remains to be determined is the precise nature of the cross-talk between the tumor and the process of skeletal muscle cancer cachexia. Certain transcript changes in the present study might be explored as biomarkers of cancer cachexia and provide insight into the nature of the process or an early indication of patient responsiveness to an intervention. We found increased serum cartilage oligomeric matrix protein (COMP) gene expression in cachectic muscle and a similar pattern of expression has been seen in muscle damage in marathon runners (44). Similarly, expression of matrix metalloproteinase 3 (MMP3), also increased in cachetic muscle, is increased in models of muscle injury (45). These findings are consistent with our suggestion that removal of cancer allows skeletal muscle to recover from net catabolism. We have recently published data detailing a role for ADIPOQ, upregulated at baseline, as a network hub in cachexia (46) suggesting that this might have a role in cancer-associated muscle atrophy or be a sensitive integrator of the net underlying processes (e.g., metabolic status of the muscle). Increased expression of the phosphoenolpyruvate carboxykinase 1 (PCK1) gene has been found in lean rodents (47) in which it has been linked to increased endurance capacity. However, our finding of increased PCK1 expression in cachetic muscle may suggest that this is representative of a possible compensatory response in the face of catabolic advantage.

Of the genes downregulated at baseline, regulator of calcineurin 1 (RCAN1) inhibits calcineurin signaling and is upregulated in muscle adapting to eccentric exercise (48) and calcineurin activation may be a key feature of muscle adaptation to endurance exercise in humans (25). Again these changes suggest that the muscle is attempting to compensate for the influence of the tumor, supporting our conclusion that muscle remodeling/turover is specifically suppressed by the presence of cancer. In rodents, inactivation of the transcription factor prospero homeobox 1 (PROX1) leads to disorganization of cardiac muscle (49). If this function was recapitulated in skeletal muscle, then reduced PROX1 expression could indicate a failure of proper muscle remodeling. Our previous analysis of gene expression in muscles of patients with cancer showed that expression of the exercise-activated genes CAMK2β and TIE1 was increased in patients with increasing weight loss (19). However, although at the follow-up time point, patients with UGIC were weight-stable from the perspective of the postoperative period, their overall weight was still lower than at the point of diagnosis. Therefore, it is possible that CAMK2β and TIE1 upregulation are indicators of muscle mass regulation, an idea consistent with the relatively clear ability of these genes to identify even modest cancer cachexia muscle changes (19). CAMK2β and TIE1 expression has been shown to be activated in multiple mouse groups, across clinical centers and the association with weight loss in this new longitudinal study suggests they are “longer term” markers of muscle status and thus potential biomarkers for weight loss in cancer cachexia. Nevertheless, the optimal biomarker would predict the potential for induction of cancer cachexia in the individual and to establish and validate such a biomarker would require much larger studies.

Current therapeutic approaches to the management of muscle wasting in cancer cachexia include such general measures as nutritional support and exercise interventions (50). Difficulties with low compliance and negligible benefit have stimulated much interest in measures to overcome hypoanabolism or reducing increased catabolism. The predominance of an activated UPP driving protein breakdown in some acute rodent models of muscle atrophy has led many to suggest that the UPP should be targeted in patients with cancer cachexia. While we cannot discount the relevance of the UPP at some point during the development of cancer cachexia (e.g., during the acute phase of a period of prolonged bed rest), the findings of the present study suggest that more emphasis should be given to the stimulation of anabolism and, in particular, blocking of the specific effect that tumor burden has on muscle signaling. The finding that changes in the transcriptome are reversible with successful removal of the cancer suggests that optimal benefit might be observed if such cachexia therapy was provided during or immediately after oncological therapy, rather than being left to a stage when both the cancer and cachexia are refractory to intervention (1).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Iain J. Gallagher, Nathan A. Stephens, Alisdair J. MacDonald, et al.


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