Metabolic signatures imaged in cancer-induced cachexia

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ABBREVIATIONS

AMIDE : A Medical Image Data Examiner
Atrogin-1 : mouse atrophy gene 1
Cho : free choline
$^{18}$FDG : $[^{18}F]$fluorodeoxyglucose
FOV : field of view
GPC : glycerophosphocholine
HDL : high density lipoprotein
HPRT1 : hypoxanthine phosphoribosyltransferase-1
IL- : interleukin-
LDL : low density lipoprotein
LMF : lipid-mobilizing factor
MAC : murine adenocarcinoma
MRS : magnetic resonance spectroscopy
MRSI : magnetic resonance spectroscopic imaging
MuRF1 : muscle RING finger 1
NS : number of scans
OSEM : Ordered Subset Expectation Maximization
PC : phosphocholine
PET : positron emission tomography
PIF : proteolysis-inducing factor
q-RT-PCR : quantitative real-time PCR
ROI : regions of interest
SCID : severe combined immunodeficient
SUV : standard uptake value
tCho : total choline
TE : echo time
TNF-$\alpha$ : tumor necrosis factor-$\alpha$
TR : repetition time
PRÉCIS

The development of therapeutic strategies to manage cachexia, a wasting disease that occurs in patients with aggressive cancer, particularly certain types such as pancreatic cancer, could benefit enormously from noninvasive imaging strategies to better understand what remains a poorly managed condition.
ABSTRACT

Cancer-induced cachexia is a complex and poorly understood life-threatening syndrome that is characterized by progressive weight loss due to metabolic alterations, depletion of lipid stores and severe loss of skeletal muscle protein. Gaining the ability to non-invasively image the presence or onset of cachexia is important to better treat this condition, to improve the design and optimization of therapeutic strategies, and to detect the responses to such treatments. In this study, we used noninvasive magnetic resonance spectroscopic imaging (MRSI) and $^{18}$F-fluorodeoxyglucose ($^{18}$FDG) positron emission tomography (PET) to identify metabolic signatures typical of cachectic tumors, using this information to determine the types and extents of metabolic changes induced by the onset of cachexia in normal tissues. Cachexia was confirmed by weight loss as well as analyses of muscle tissue and serum. In vivo, cachexia-inducing MAC16 tumors were characterized by higher total choline (tCho) and higher $^{18}$FDG uptake compared to histologically similar non-cachectic MAC13 tumors. A profound depletion of the lipid signal was observed in normal tissue of MAC16 tumor bearing mice but not within the tumor tissue itself. High-resolution $^1$H MR spectroscopy (MRS) confirmed the high tCho level observed in cachetic tumors that occurred due to an increase of free choline and phosphocholine (PC). Higher succinate and lower creatine levels were also detected in cachetic tumors. Taken together, these findings enhance our understanding of cancer's effect on host organs and tissues as well as promote the development of noninvasive biomarkers for the presence of cachexia and identification of new therapeutic targets.
INTRODUCTION

Cachexia is a complex metabolic and nutritional syndrome characterized by a massive loss of adipose tissue and skeletal muscle (1). The term cachexia is coined from the Greek words ‘kakos’ and ‘hexis’ for ‘bad condition’ (2). Cachexia is encountered not only in cancer, but also in other life-threatening diseases such as acquired immunodeficiency syndrome, rheumatoid arthritis, chronic obstructive pulmonary disease, and organ failure (1). In cancer patients, the cachectic syndrome is a major cause of morbidity and mortality. Progressive cachexia indicates poor prognosis with a shorter survival time, and ultimately accounts for nearly 20% of all cancer deaths (3, 4). The rate and amount of weight loss are directly related to survival in cancer patients (5). Cachexia occurs with a frequency of 83 to 87% in patients with pancreatic and gastric cancer, 48 to 61% in patients with cancers of the colon, prostate, lung, head and neck, and unfavorable non-Hodgkins lymphoma, and 31 to 40% in patients with breast cancer, sarcomas, leukemia, and favorable subtypes of non-Hodgkins lymphoma (4).

Cachexia involves the secretion of tumor-derived catabolic factors along with host-derived pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β and interferon-γ (IFN-γ). Tumor-derived catabolic factors such as lipid-mobilizing factor (LMF) lead to changes in host metabolism and energy expenditure, resulting in cancer cachexia (6). LMF is a toxohormone that up-regulates degradative pathways in adipose tissue, and increases fat metabolism in cachectic animals and humans (6). Glucocorticoids and tumor-derived proteolysis-inducing factor (PIF)
also play critical roles in this condition (7). The consequences of the significant weight loss and the massive loss of proteins are multiple and severe, ranging from impairment of immune function to poor outcome of chemotherapy, fatigue, respiratory muscle weakness and markedly reduced quality of life (4, 5).

There is limited understanding of the pathogenesis of this lethal syndrome, and no known cure, since mechanisms underlying its manifestation are not defined clearly enough to design effective therapeutic strategies. As a result therapy is ineffective, and there is an urgent need to identify new targets to develop effective therapies. The ability to detect the onset or presence of cachexia non-invasively, and detect response to treatment, is central to developing new therapies. Significant abnormalities in carbohydrate, lipid and protein metabolism are observed with cachexia, and are a major cause for the associated profound weight loss (5).

Non-invasive clinically translatable techniques such as magnetic resonance spectroscopy (MRS) and positron emission tomography (PET) with their respective metabolic imaging capabilities provide the opportunity to investigate the interaction between cancer cells and the tumor, the ‘tumor macroenvironment’ to identify stages in the cachexia-cascade. With its clinical translatable, relatively high sensitivity and ability to detect metabolites such as lipids, lactate, and total choline (tCho), which consists of free choline (Cho), phosphocholine (PC) and glycerophosphocholine (GPC), $^1$H MRS imaging (MRSI) is ideally suited to explore the interactions between the tumor and its host in the induction and progression of cachexia. Similarly, $[^{18}\text{F}]$fluorodeoxyglucose ($^{18}$FDG) positron
emission tomography (PET) is frequently used in oncological diagnosis. Here we have
identified tumor and normal tissue metabolic signatures associated with the well-
established cachexia-inducing murine adenocarcinoma (MAC) 16 tumor model,
compared to the histologically similar but non-cachectic MAC13 tumor model.
The presence of cachexia in our experimental model was confirmed by weight loss and
an increase in the expression levels of two markers of muscle protein degradation,
Atrogin-1 (mouse atrophy gene 1) and MuRF1 (muscle RING finger 1). Atrogin-1 and
MuRF1 are two skeletal muscle specific ubiquitin ligases required in protein breakdown
(8), and the expression levels of these ligases increases with cachexia, and (5, 8-10). The
presence of cachexia was additionally confirmed by the analysis of lipid content in serum
from cachectic and non-cachectic tumor bearing mice (11, 12).
In vivo, cachectic tumors were characterized by increased tCho and $^{18}$FDG uptake
compared to non-cachectic ones, and induced a profound depletion of triglycerides in
normal tissue that was not observed with non-cachectic tumors. Cachexia also resulted in
increased $^{18}$FDG uptake in the brain and lung. An increase of PC and succinate along
with a decrease of creatine were detected in high-resolution spectra of cachectic tumor
extracts. The higher choline kinase mRNA levels observed in MAC16 cells and tumors
were consistent with higher PC observed in tumor extract spectra and the increased tCho
observed in vivo. These metabolic patterns may represent new noninvasive biomarkers
and targets in the detection, management, and treatment of this condition.
MATERIALS AND METHODS

Cell lines and tumor models. Cachectic MAC16 and non-cachectic MAC13 cell lines were obtained from Dr. Sidransky at Johns Hopkins University with Dr. Tisdale’s permission. Cachectic MAC16 tumors induce significant weight loss in tumor-bearing animals, unlike MAC13 tumors, which although histologically similar, do not alter body weight (13). Cells were cultured in RPMI 1640 with 10% fetal bovine serum. Tumors were generated by inoculating a cell suspension of 2 x 10^6 cells in 0.05 ml of Hanks balanced salt solution in the flank of severe combined immunodeficient (SCID) male mice. Tumor volumes were calculated from caliper measurements using the equation, volume = (π/6) × a × b × c, where a, b, and c represent three orthogonal axes of the tumor. Since these are murine cancer cell lines, authentication with commercially available kits that are used for human cancer cell lines is not possible. Instead, tumor-induced weight loss, expression of muscle degrading markers, and serum lipid profiles were used to authenticate the cells.

FDG PET imaging. For 18F-fluorodeoxyglucose (FDG) PET imaging, MAC13 and MAC16 tumor-bearing mice were fasted overnight. Mice were injected intravenously (i.v.) with 200 μCi of FDG in a final volume of 200 μl. At 60 minutes post-injection of the radiotracer, a 30-minute static whole-body image was acquired over the mouse body. Images were decay corrected and reconstructed using 2D OSEM (Ordered Subset Expectation Maximization). Data analysis was performed based on regions of interest (ROI) drawn over the tumor and tissues. After accounting for injected dose and body
weight, all radioactivity concentration values were converted into Standardized Uptake Values (SUV). Values shown are means of the pixel values (SUV\text{mean}). In the case of tumor, to avoid bias from the inclusion of any tumor necrotic areas in the ROI, SUV\text{mean} as well as SUV\text{max} (single maximum pixel value within the ROI) were reported. Image analysis was performed using AMIDE (A Medical Image Data Examiner) software (SourceForge).

**In vivo $^1$H MR spectroscopic imaging (MRSI).** Anesthetized mice bearing tumor xenografts were imaged on a 4.7T Bruker Biospec spectrometer (Bruker Biospin Corp., Billerica, MA). Body temperature of the animals was maintained in the magnet by a thermostat-regulated heating pad. To acquire localized proton spectra of the tumor, a home-built solenoid coil was placed around the tumor. Spectra from a 4 mm thick slice were acquired with a field of view (FOV) of 16 mm; a matrix size of 16 x 16 x 1024; 4 scans per phase encode step; number of scans (NS) of 4, an echo time (TE) of 120 ms and a repetition time (TR) of 1 s, using a 2D-CSI sequence with VAPOR water suppression (14). Signals at ~ 3.2 ppm from tCho and at 1.3 ppm from lipids with some contribution from lactate that overlaps with the lipid signal were detected in localized proton spectra. A home-built volume coil was used to acquire whole-body $^1$H MR spectroscopic images and obtain in vivo cross-sectional images from the tCho and lactate+lipids signal of the mouse. Spectra from a 4 mm thick slice were acquired with a FOV of 32 mm; a matrix size of 32 x 32 x 1024; 4 scans per phase encode step; NS = 4, TE = 120 ms and TR = 1 s. Reference 2D-CSI images of the unsuppressed water signal were acquired with TE = 20 ms and NS = 1, with all other parameters remaining the same.
Quantitative maps of tCho and lactate+lipids were generated from the spectroscopic images using unsuppressed water signal as an internal reference (15). Quantitative metabolic maps were generated using in-house IDL programs.

**High-resolution $^1H$ MR spectroscopy of dual phase tissue extracts.** High-resolution $^1H$ MR spectroscopy of lipid- and water-soluble fractions of tissue extracts was performed to resolve the components of the tCho signal and the lactate+lipids signal detected *in vivo* and further characterize the differences observed *in vivo*. Lipid- and water-soluble fractions were obtained from tumors and muscle tissue using a dual-phase extraction method with methanol/chloroform/water (1/1/1) (16). Briefly, tissues were freeze-clamped and ground to powder. Ice-cold chloroform, followed by ice-cold water, was added and the sample was kept at 4°C overnight for phase separation. Samples were then centrifuged for 30 minutes at 15,000 g at 4°C to separate the phases. The water/methanol phase containing the water-soluble metabolites was treated with chelex (Sigma Chemical Co., St Louis, MO) for 10 minutes on ice to remove divalent cations. Methanol was then removed by rotary evaporation, and the remaining water phase was lyophilized and stored at -20°C. The chloroform phase containing the lipids was dried in a stream of N$_2$ and stored at -20°C. Water-soluble samples were dissolved in 0.5 ml of D$_2$O (Sigma Chemical Co., St Louis, MO) containing 3-(trimethylsilyl) propionic-2,2,3,3,-d$_4$ acid (Sigma Chemical Co., St Louis, MO) as an internal concentration standard (sample pH of 7.4). Lipid samples were dissolved in 0.6 ml of CDCl$_3$/CD$_3$OD (2/1) containing tetramethylsilane as an internal concentration standard (CDCl$_3$ and CD$_3$OD premixed with tetramethylsilane by the manufacturer, Cambridge Isotope Laboratories, Inc.). Fully
relaxed $^1$H MR spectra of the extracts were acquired on a Bruker Avance 500 spectrometer operating at 11.7 T (Bruker BioSpin Corp., Billerica, MA) using a 5-mm HX inverse probe and the following acquisition parameters: 30° flip angle, 6000 Hz sweep width, 12.7 s repetition time, time-domain data points of 32K, and 128 transients (16). Spectra were analyzed using Bruker XWIN-NMR 3.5 software (Bruker BioSpin). Integrals of the metabolites of interest were determined and normalized to the tumor weight. To determine concentrations, peak integration from $^1$H spectra for all metabolites studied was compared to the internal standard.

**Quantitative real-time PCR of cells and tumor extracts.** Total RNA was extracted from MAC13 and MAC16 cells and tumors, using Qiagen easy mini kit (Qiagen, CA, USA) as previously described (17). Complementary DNA (cDNA) was synthesized from 1μg of total mRNA using qScript (Quanta Bioscience, MD, USA). Quantitative real-time PCR (q-RT-PCR) was performed using iQ SYBR Green Supermix, gene-specific custom-made primers, and diluted cDNA (1:10) samples as template, in the iCycler RT-PCR detection system (Bio-Rad, CA, USA). The following primers were designed using Beacon Designer software 5.1 (Premier Biosoft International, CA, USA): mChk (NM_013490) forward 5’-CCAGTTCCACATCAGTGTCATCAG-3’, reverse 5’-CTCTCCAGAACCATCGCCTCAG-3’; hypoxanthine phosphoribosyltransferase-1 (HPRT1) (NM_000194) forward 5’-CCTGGCGTCGTGATTAGTGATG-3’, reverse 5’-CAGAGGGCTACAATGTGATGGC-3’ (annealing temperature, 60°C). The expression of the target RNA relative to the housekeeping gene HPRT1 was calculated based on the
threshold cycle (Ct) as \( R = 2^{-\Delta(\Delta C_t)} \), where \( \Delta Ct = C_t target - C_t HPRT1 \) and \( \Delta(\Delta Ct) = \Delta C_t MAC16 - \Delta C_t MAC13 \).

Quantitative real-time PCR of muscle tissue. Total RNA was extracted from the gastrocnemius muscle of mice bearing MAC13 or MAC16 tumors using Trizol (Invitrogen, Carlsbad, USA). Briefly, the muscle tissue was freeze-clamped, ground to powder, and 2 ml of Trizol added per 250 to 400 mg of ground tissue and homogenized. Further steps to purify RNA were carried out following the manufacturer’s recommendation (Invitrogen). cDNA, q-RT-PCR and determination of the fold changes were performed as described previously. The following mouse specific primers at optimized annealing condition were used: Tripartite motif-containing 63 (Trim63/MuRF1, NM_001039048), forward- 5’-AAGACTGAGCTGAGTAACTG-3’, reverse-5’-TAGAGGGTGTCAAACTTCTG-3’, (annealing temperature, 56.1°C); F-box protein 32 (Atrogin-1, NM_026346), forward- 5’-AGAAAGAAAGACATTCAGAACA-3’, reverse-5’- GCTCCTTCGTACTTCTCTT-3’, (annealing temperature, 54°C).

Protein isolation and western blot analysis of muscle tissue. Total protein from freeze-clamped muscle tissue was extracted using RIPA buffer fortified with protease inhibitor cocktail (1/500, SIGMA), dithiothreitol (1/1000, 1M stock), phenylmethylsulfonyl fluoride (1/200, 0.2M stock), sodium orthovanadate (1/500, 0.5M stock) and sodium fluoride (1/500, 0.5M stock). About 100 μg of protein was resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with a goat polyclonal antibody (1/1000 in 5% non-fat dry milk) against Atrogin-1 (Everest biotech, Oxfordshire, UK) or
rabbit polyclonal (1/1000 in 5% non-fat dry milk) against MuRF1 (Novus Biologicals, Littleton, Co, USA). Appropriate horseradish peroxidase (HRP) conjugated secondary antibody donkey anti-goat (Novus Biologicals, Littleton, Co, USA) or donkey anti-rabbit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) were used at 1/2000 dilution. A mouse monoclonal antibody against GAPDH (Sigma) at 1/1000 was used as loading control. Immunoblots were developed using SuperSignal West Pico chemiluminescent substrate kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

**Serum analysis.** Lipid analysis of serum was performed by the Johns Hopkins Phenotyping and Pathology Core. Lipids measured included cholesterol, triglycerides, LDL (low density lipoprotein) and HDL (high density lipoprotein).

**Statistical analysis.** Values displayed are mean ± SEM. Statistical significance was evaluated using the Student’s t-test; $P < 0.05$ was considered significant.

**RESULTS**

**Confirming the presence of cachexia.** Cachectic MAC16 and non-cachectic MAC13 tumors displayed comparable growth rates and histology. However, as shown in Figure 1a, MAC16 tumors induced significant weight loss, confirming the induction of cachexia. MAC16 and MAC13 tumor-bearing mice that were imaged weighed $22.29 \pm 0.89$ g and $25.41 \pm 0.55$ g ($P < 0.05$) respectively. Tumor volumes at $535.8 \pm 44.3$ mm$^3$ for the MAC16 and $493.8 \pm 40.6$ mm$^3$ for MAC13 tumors were not significantly different. The
mRNA (Figure 1b) and protein expression levels (Figure 1c) of MuRF1 and Atrogin-1, which are markers of muscle degradation, were significantly higher in muscle tissue of mice bearing MAC16 tumors compared to MAC13 tumors. Serum from cachectic mice was characterized by significantly lower triglycerides and higher LDL (Figure 1d). Total cholesterol and HDL levels were not significantly different.

*Cachectic tumors show higher glucose uptake than non-cachectic tumor and induce increased uptake of glucose in brain and lungs.* Representative images shown in Figure 2a demonstrate the increased uptake of $^{18}$FDG in MAC16 compared to MAC13 tumors. Quantification of $^{18}$FDG uptake confirmed a significant difference between MAC13 and MAC16 tumors (Figure 2b). $^{18}$FDG uptake in the brain (Figure 2c) and lung (Figure 2d), but not muscle (Figure 2e), was significantly higher in mice bearing cachectic tumors.

*Cachectic tumors have altered total choline levels in vivo.* Representative high-resolution $T_1$-weighted images of a MAC13 (upper panel) and MAC16 (lower panel) presented in Figure 3a demonstrate that the MAC16 (lower panel) tumor had significantly higher tCho compared to the MAC13 tumor (upper panel), as evident in the tCho maps shown in Figure 3b. Representative lactate+lipids images from these tumors are shown in Figure 3c and demonstrate that, although the lactate+lipids content was not different between the MAC13 (upper panel) and the MAC16 (lower panel) tumor, the peripheral signal, most likely from subcutaneous lipids, was lower around the MAC16 tumor.
Quantitative tCho and lactate+lipids data acquired from multiple animals are summarized in Figures 3d-f. A significant increase of tCho was observed in MAC16 compared to MAC13 tumors (Figure 3d). There was no significant difference in lactate+lipids obtained from within the tumor (Figure 3e), but a significant decrease of lactate+lipids around the tumor periphery in MAC16 compared to MAC13 tumors (Figure 3f). The lactate+lipids signal around the periphery of the tumor was from subcutaneous fatty tissue that is found between the tumor and the fold of the skin, which decreased as the lipids decreased in normal tissue of cachectic mice, as discussed subsequently.

**Cachectic tumor induces lipid depletion in normal tissue.** To determine the effect of cachectic tumors on normal tissue tCho and lactate+lipids, we performed *in vivo* $^1$H MRSI of the mouse whole body using a volume coil. Representative high-resolution T$_1$-weighted images of a MAC13 (upper panel) and MAC16 (lower panel) are presented in Figure 4a to identify the cross-sectional area from which the metabolite images were obtained. Representative lactate+lipids images obtained from this cross-sectional slice are shown in Figure 4b and demonstrate the profound depletion of lactate+lipids signal in normal tissue in MAC16 tumor-bearing mice (lower panel) compared to MAC13 tumor-bearing mice (upper panel), as evident in Figure 4c that displays the lactate+lipids signal overlaid with the spin-density images.

Quantitative lactate+lipids data acquired from multiple animals are summarized in Figure 4d. A significant decrease of lactate+lipids was observed in the normal tissue of MAC16 tumor-bearing mice compared to MAC13 tumor-bearing mice that was subsequently
identified to be due to lipid depletion. No significant differences in tCho were observed in the normal tissues of MAC16 and MAC13 tumor-bearing mice.

**Tumor extract studies confirmed in vivo data.** To characterize better the observations made *in vivo*, we performed high-resolution proton spectroscopy of water-soluble and lipid extracts of tumors. The tCho peak observed *in vivo* consists of free choline, PC and GPC that can be identified in high-resolution spectra of water-soluble extracts. Similarly, contributions from lactate versus lipids to the lactate+lipids signal observed *in vivo* can be resolved since lactate is separated into the water-soluble phase and the lipids are separated into the lipid phase of the tumor extracts. Representative spectra from water-soluble tumor extracts are shown in Figure 5a and b, and demonstrate significantly higher PC and free choline in MAC16 (Figure 5b) tumors compared to MAC13 tumors (Figure 5a), but no difference in lactate. Quantitative data acquired from multiple tumors are summarized in Figure 5c and demonstrate a significant increase of free choline, PC, and total choline that confirmed the *in vivo* data.

Additionally, a significant increase of succinate and a significant decrease of creatine were observed in the water-soluble tumor extracts of MAC16 compared to MAC13 tumors (Figure 5c). There were no significant differences in phosphocreatine, alanine or acetate (data not shown) between MAC16 and MAC13 tumors. Lipid signals in the lipid-phase extracts were not significantly different between MAC16 and MAC13 tumors again confirming the *in vivo* data (data not shown).
To identify further the molecular mechanism underlying the increase of PC we performed quantitative RT-PCR of choline kinase, the enzyme that phosphorylates free choline to PC. We found a significant increase of choline kinase mRNA in MAC16 cells (Figure 6a) and tumors (Figure 6b).

**Cachexia affects lipid content of muscle.** The profound depletion of lipids in normal tissue observed *in vivo* was confirmed in high-resolution spectra of extracted muscle tissue. Representative lipid spectra from muscle tissue extract obtained from MAC13 and MAC16 tumor bearing mice are shown in Figure 7a and b respectively. In the spectra, the different peaks are assigned to the protons signals of different groups of fatty acids, methyl (-CH$_3$, 0.9 ppm), aliphatic methylene (-CH$_2$, 1.3 ppm), carboxyl (-OOC-CH$_2$-CH$_2$-, 1.47 ppm; -OOC-CH$_2$, 2.4 ppm), allyl methylene (=CH-CH$_2$, 1.92 ppm), vinyl (=CH-, 5.3 ppm) (18-20). Signals from saturated lipids are visible at 1.3 and 0.9 ppm, poly-unsaturated lipids at 1.92 and 5.3 ppm and the carboxyl group attached to fatty acids at 1.47 and 2.4 ppm. Quantitative data summarized from multiple animals are shown in Figure 7c and demonstrate the significant decrease of lipids induced in the muscle by cachexia. Signals from all of these groups, but especially the carboxyl and methyl groups, decreased in the muscle tissue extracted from cachectic mice.

**DISCUSSION**

Here we have identified, with noninvasive imaging, clinically translatable metabolic signatures in tumors and normal tissue that potentially detect the presence of cachexia.
Cachectic MAC16 tumors displayed increased tCho and $^{18}$FDG uptake compared to non-cachectic MAC13 tumors. A profound depletion of the lipid signal was observed in normal tissue of mice bearing cachexia-inducing MAC16 tumors, in addition to increased $^{18}$FDG uptake in the brain and lungs. High-resolution spectroscopy of tissue extracts identified additional metabolite signatures, such as increased PC and succinate, and reduced creatine, which may also provide targets to reverse or inhibit the cachexia cascade.

The presence of cachexia induced by MAC16 tumors was confirmed by the significant weight loss, the increase of MuRF-1 and Atrogin-1 mRNA and protein expression in muscle tissue, and a significant decrease of triglycerides and increase of LDL in serum of MAC16 tumor bearing mice. MuRF-1 and Atrogin-1 have been identified as muscle protein breakdown markers that increase with cachexia (10). Atrogin-1 and MuRF1 are skeletal muscle specific ubiquitin ligases involved in the ubiquitin proteasome pathway and essential in the development of muscle atrophy (8, 9). Decreased serum triglycerides and increased LDL have also been previously associated with cachexia (11, 12). Collectively these data confirmed the induction of cachexia in MAC16 tumor-bearing mice.

Elevated tCho is characteristic of malignant tumors compared to normal tissue, but here we observed a two-fold increase of tCho in cachectic MAC16 tumors compared to non-cachectic MAC13 tumors, suggesting that tumors with higher than usual tCho may pose a risk for the induction of cachexia. The increased tCho was identified to be due to
significantly higher PC and free choline. An increase of cellular PC and choline-containing compounds has been consistently observed in cancer cells and tissue (21-23), and is closely related to malignant transformation, invasion, and metastasis (24, 25). Elevated PC and tCho have been previously detected by MRS studies in human lung, colon, prostate, and breast tumors, as well as in derived epithelial cell lines (21, 23). PC is a precursor as well as a metabolic breakdown product of the major membrane component phosphatidylcholine. Here we have identified a previously unknown association between choline metabolism and cachexia. We found that mRNA for choline kinase, a cytosolic enzyme that catalyzes the phosphorylation of choline to PC by ATP in the presence of magnesium, was significantly elevated in MAC16 cells and tumors, identifying it as a potential novel target to investigate in the treatment of cachexia.

Increased $^{18}$FDG uptake is used to detect metabolically active cancers. $^{18}$FDG is taken up by the cells via glucose transporters and phosphorylated by the first enzyme of the glycolysis, the hexokinase. $^{18}$FDG PET can be used in vivo to identify tumor tissues, since cancer cells exhibit a higher glycolytic activity, leading to a higher uptake of $^{18}$FDG (26). Here we found that similar to tCho, $^{18}$FDG uptake was more than two-fold higher in cachectic MAC16 tumors compared to non-cachectic MAC13 tumors, suggesting that exceedingly high $^{18}$FDG uptake may be another risk factor for the induction or presence of cachexia. Although high $^{18}$FDG uptake is usually associated with increased glycolytic activity (26, 27), we did not observe a significant difference in lactate levels between MAC13 and MAC16 tumors. Interestingly we did detect a higher level of succinate in the cachectic tumors. Since succinate is produced in the tricarboxylic acid cycle (TCA)
cycle from α-ketoglutarate (28), increased flux through the TCA cycle may explain a higher uptake of glucose without an increase of lactate.

Subcutaneous lipids located around the tumor were significantly reduced in the cachectic tumor-bearing mice and lipids in surrounding normal tissue and the skeletal muscle of MAC16 tumor-bearing mice were depleted. It has been previously shown that increased adipocyte lipolysis, rather than decrease in synthesis, is responsible for the loss of fat mass with cachexia (29). Moreover acetyl-CoA required for the TCA cycle can be obtained from degradation of lipids. The decrease in lipids observed in cachectic mice may arise from an increase in the flux through the TCA cycle, which is also consistent with the increase of succinate. Depletion of lipids in the muscle can be easily detected clinically with $^1$H MRS, and may be of value in detecting the presence or onset of cachexia or in evaluating the effect of treatments arresting or reversing this condition.

The search for noninvasive biomarkers of cachexia has been ongoing for several years. One $^1$H MRS-based search for cachexia biomarkers in the sera of C26 adenocarcinoma-bearing mice identified very low-density lipoproteins along with hypoglycemia as serum markers (11). An assessment of hepatic and skeletal muscle bioenergetic status using $^{31}$P MRS demonstrated that progressive cancer cachexia was associated with depletion of energy stores in liver and skeletal muscle (30). Increased hepatic Pi/ATP ratio was detected by $^{31}$P MRS, and occurred early in the disease process (30). The liver is a central organ in the pathogenesis of cachexia (31). Increased Pi and a rapid removal of phosphomonoesters were identified with $^{31}$P MRS in a rat cancer model of cachexia.
Cachexia also induced enhanced hepatic gluconeogenic activity (32). While the use of $^{31}$P MRS is less practical for clinical applications due to the poor sensitivity of detection limiting spatial localization, detection of global changes in liver metabolism with $^{31}$P MRS may provide additional specificity in detecting the onset or presence of cachexia.

In conclusion we have, in a preclinical model, identified previously unknown clinically translatable tumor and normal tissue metabolic signatures characteristic of cachexia. The ease of translating these signatures to the clinic should facilitate validating these metabolites as potential biomarkers of cachexia. In addition, the metabolic changes identified here provide new targets to exploit in the treatment of cachexia. Future studies with noninvasive imaging will allow us to establish a sequence of events to identify the most lethal metabolic aspects of the cascade. Once critical single or multiple cachectic switches or cascades are identified, molecular targeted image-guided approaches such as multiple siRNA targeting or pharmaceutical interventions can be designed and developed to down-regulate or knockout effector molecules, pathways or enzymes controlling the cascade.
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LIST OF REFERENCES


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FIGURE LEGENDS

Figure 1: (a) Normalized weights of MAC13 (◆) and MAC16 (■) tumor-bearing mice over time. (n = 10 per group, *P < 0.05). (b) mRNA expression levels of MuRF1 and Atrogin-1 analyzed by RT-PCR in MAC13 (■) and MAC16 (■) muscle tissue. Values represent Mean ± SEM (n = 4, **P < 0.01, ***P < 0.005). (c) Representative immunoblot showing MuRF1 and Atrogin-1 proteins in muscle tissue obtained from mice bearing from MAC13 or MAC16 tumors. GAPDH was used as loading control (n = 3). (d) Lipid analysis of serum from mice bearing MAC13 or MAC16 tumors. Values represent Mean ± SEM (MAC13, n = 8, MAC16, n = 7; *P < 0.05, **P < 0.01).

Figure 2: (a) Representative PET images of 18FDG uptake in MAC13 and MAC16 tumors (SUV: standardized uptake value). Quantification of the uptake in (b) tumors, (c) brain, (d) lungs and (e) muscle. Values represent Mean ± SEM (MAC13, n = 8, MAC16, n = 5; *P < 0.005).

Figure 3: Representative (a) T1-weighted images, (b) tCho maps and (c) lactate+lipids maps of a MAC13 (upper panel) and a MAC16 (lower panel) tumor. T1-weighted images were acquired from the corresponding 4 mm slice used for MRSI using a spin-echo sequence with an echo time of 10 ms, a repetition time of 500 ms, and an in-plane spatial resolution of 62.5 μm. Total choline maps and lactate+lipids maps were generated from the MRSI data and normalized to the water signal to display concentrations in mM units. (d) In vivo tCho concentrations in MAC13 and MAC16 tumors. (e) In vivo lactate+lipids concentration within MAC13 and MAC16 tumors. (f) In vivo concentration of
subcutaneous lipids surrounding MAC13 and MAC16 tumors. Values represent Mean ± SEM (n = 10 per group, * P < 0.05).

**Figure 4:** (a) Cross-sectional T₁-weighted images, (b) cross-sectional lactate+lipids maps, and (c) merged images from (a) and (b) of MAC13 (upper panel) and MAC16 (lower panel) tumor-bearing mice. T₁-weighted images were acquired from the corresponding 4 mm slice used for MRSI using a spin-echo sequence with an echo time of 10 ms, a repetition time of 500 ms, and an in-plane spatial resolution of 125 μm. Lipid maps were generated from MRSI data and normalized to the water signal. Volumes were comparable for the MAC13 (540 mm³) and MAC16 (545 mm³) tumors. (d) *In vivo* lactate+lipids concentration in MAC13 (■) and MAC16 (■) tumor-bearing mice (n = 6 per group). Values were obtained from a 4 mm axial slice using a volume coil. Values represent Mean ± SEM. * P < 0.05.

**Figure 5:** Representative ¹H MR high-resolution spectra of water-soluble (a) MAC13 and (b) MAC16 tumor extracts. Cho (free choline), PC (phosphocholine), GPC (glycerophosphocholine). (c) Concentration of metabolites (Cho, PC, GPC, total choline = Cho+PC+GPC, succinate and creatine) in MAC13 (■) and MAC16 (■) tumors (n = 6 per group, * P < 0.05, ** P < 0.001). Values represent Mean ± SEM.

**Figure 6:** mRNA levels of Chk analyzed by RT-PCR in MAC13 (■) and MAC16 (■) (a) cell and (b) tumor extracts. Values represent Mean ± SEM (n = 5 per group for the cell extract, n = 8 per group for the tumor extracts, * P < 0.05, *** P < 0.005).
**Figure 7:** Representative $^1$H MR high-resolution spectra of lipid-soluble muscle extracts obtained from (a) MAC13 and (b) MAC16 tumor bearing mice. The chemical shift reference peak is tetramethylsilane that is also used as an internal concentration standard. (c) Quantification of lipids in arbitrary units from lipid-soluble extracts of muscle tissue from MAC13 (■) and MAC16 (■) tumor bearing mice (n = 3 per group). Values represent Mean ± SEM. * $P < 0.05.$
Figure 1

(a) Normalized Mouse Weight (%)

(b) mRNA expression level

(c) Western Blot

(d) Serum Lipids
Figure 2

- **a** Tumor SUV images for MAC13 and MAC16.
- **b** Box plots showing mean and max SUV for MAC13 and MAC16.
- **c** SUV values for Brain in MAC13 and MAC16.
- **d** SUV values for Lungs in MAC13 and MAC16.
- **e** SUV values for Muscle in MAC13 and MAC16.
Figure 3

d

![Graph](image)

e

![Graph](image)

f

![Graph](image)
Figure 4
Figure 5

(a) GPC/PC/Cho

(b) GPC/PC/Cho

(c) Molar concentration (mM) of different compounds

- Cho
- PC
- GPC
- Total choline
- Succinate
- Creatine
Figure 6

(a) Cell extract (b) Tumor extract

Chk mRNA expression level

***(***
Figure 7

(a) 

3.0 2.0 1.0 0.0 ppm

5.0 4.0 3.0 2.0 1.0 0.0 ppm

-CH=CH-

-OOC-CH2-CH2

-CH2-CH2-CH=

-OOC-CH2-

CH3

Reference

(b) 

3.0 2.0 1.0 0.0 ppm

5.0 4.0 3.0 2.0 1.0 0.0 ppm

-CH=CH-

-OOC-CH2-CH2

-CH2-CH2-CH=

-OOC-CH2-

CH3

Reference

(c) 

Arbitrary units

2500

2000

1500

1000

500

* *

CH3 (CH2)n OOC-(CH2)2 (CH2)2-CH= OOC-CH2 CH=CH
Metabolic signatures imaged in cancer-induced cachexia

Marie-France Penet, Mayur Mahavir Gadiya, Balaji Krishnamachary, et al.

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