Supplemental Materials

Brain Metabolites in Cholinergic and Glutamatergic Pathways are Altered by Pancreatic Cancer Cachexia

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Running Title: Cachexia brain metabolic signature

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Supplemental Methods

H&E staining of brain tissue. Mouse brains were rapidly removed at sacrifice, bisected sagittally and each half was placed into formalin for a 24 – 36 h fixation at 4^{o} C. Formalin fixed brains were rinsed with PBS and stored in 70% ETOH prior to delivery to a Johns Hopkins Core facility where the samples were embedded in paraformaldehyde, sectioned (5-7 μm) onto slides, and stained with H&E, using standard protocols.

Protein extraction, protein concentration estimates, SDS-PAGE, and immunoblots. Snap-frozen brain tissue samples (30 – 40 mg) were pulverized under liquid N_2 and upon reaching ~4^{o} C lysed with 5 volumes of RIPA buffer solution supplemented with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) along with 1 mM each of DTT, sodium orthovanadate, sodium fluoride, and phenylmethylsulfonyl fluoride. Cell lysis/protein extraction proceeded for 30 min on ice followed by sonication and centrifugation at 12,000 rpm for 30 mins at 4^{o}C. Cleared supernatants were decanted into fresh tubes and stored at -80^{o} C.

Protein concentration estimates were determined using a Bradford protein assay kit (Pierce, Rockford, IL, USA) according the manufacturer's instructions.

SDS-PAGE: Proteins (60-100 μg) were resolved in 7% gels during electrophoresis at room temperature and constant voltage (100 V) for 3 h. Proteins were transferred to nitrocellulose membranes at 40-mA current at 4^{0}C overnight.

Immunoblots: Antibodies used to determine the expression level of various protein in the samples from each cohort are listed in Supplemental Table 1. Immediately following the transfer, membranes were stained with Ponceau Red to check for quality of transfer and later washed in water followed by TBST for 5 min. The membrane was blocked in 5% skim milk-TBST for 1 hour and probed overnight at 4^{o} C with the appropriate antibody diluted in 5% skim milk-TBST

(Supplemental Table 1). In all cases, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control and probed with monoclonal anti-GAPDH antibody (1:50,000 dilution, Sigma-Aldrich, St Louis, MO) in 5% skim milk-TBST for 2 h at room temperature. The membranes were then washed 3x 15 min in TBST and probed with appropriate (either anti-rabbit or anti-mouse IgG) horseradish peroxidase-labeled secondary antibodies (GE health care, Piscataway, NJ, USA) in 5% skim milk-TBST for 1 h at room temperature. Blots were developed using a SuperSignal West Pico kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol and visualized using X-Ray film.

Human ^{1}H MR spectroscopic imaging

Clinical multivoxel MR spectroscopic imaging (MRSI) was performed using a Siemens Prisma 3T and a 20-channel head coil, a single 10 mm thick slice using a Stimulated Echo Acquisition Method (STEAM) centered in the midbrain, just above the corpus callosum in deep white matter. Prior to MRSI, shimming was performed to optimize field homogeneity, and water suppression was optimized using automated routines provided by the manufacturer. The scanning parameters were: TR/TM/TE 1700/10/20ms, 16×16 matrix size, slice thickness 10 mm, 16×16 cm field of view, total data acquisition time was approximately nine minutes, and nominal voxel size was 1.0 ml. The echo signal was digitized with 2048 data points and a spectral width of 1,200 Hz. Raw spectroscopy data were processed using MRSI software (syngo MR B17). The spectra were used to calculate relative metabolite concentrations. Anatomical T1 MPRAGE image (TE/TR = 2.9/2000 ms), T2-weighted (TE/TR = 96/7270ms) and FLAIR (TE/TR/IR=89/9000/2500ms) images were acquired for each subject.

Supplemental Table 1. Primary antibodies used at the given dilutions and the suppliers.

Antigen	Antibody Type	Dilution	Supplier	Catalogue #
Choline Kinase-α (Chk-α)	Rabbit Polyclonal	1:1000	Abcam	Ab88053
Choline Transporter-1 (CHT1/SLC5A7)	Rabbit Polyclonal	1:1000	Abnova	PAB5294
Choline Transporter-like-1 (CTL1/SLC44A1)	Rabbit Polyclonal	1:1000	ABclonal	A15413
Glutamine Synthetase (GLUL)	Rabbit Polyclonal	1:40,000	Biorbyt	P15104
Glutaminase 1 (GLS1)	Rabbit Monoclonal	1:1000	Invitrogen	6H5L15
Glutaminase 2 (GLS2)	Rabbit Polyclonal	1:500	GeneTex	GTX133243