Supporting Online Material for

Adipose Triglyceride Lipase Contributes to Cancer-Associated Cachexia

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Methods

Animal care
Female C57BL/6 mice were maintained on a regular light-dark cycle (12 hours (h) light, 12 h dark) and kept on a standard laboratory chow diet (4.5 % w/w fat). Hsf⁻/⁻ and Atgl⁻/⁻ mice were generated by targeted homologous recombination (1-2). Mice used for injection of tumor cells as well as control animals were 8-9 weeks of age. During experiments, food intake as well as body weight was monitored. All studies were performed with littermates of the same genetic background as controls. All animal studies were performed in accordance with the guidelines and provisions of the Commission for Animals Experiments of the Austrian Ministry of Science and recommendations of the local ethics committee.

Tumor implantation and tissue harvesting
Mice were anesthetized using isoflurane and blood samples were collected by retro-orbital puncture. Animals were randomly divided into groups. 4x10⁶ LLC cells or 2x10⁶ B16 melanoma cells, obtained from an exponentially proliferating cell culture, maintained in conventional Dulbecco’s Modified Eagle Medium (DMEM) high glucose cell culture medium containing 10% fetal bovine serine (FBS) [v/v] (PAA, Pasching, Austria), or saline solution as negative control were injected subcutaneously at dorsum just below the neck. After 14 or 21 d (for LLC) and 16 d (for B16 melanoma cells), o/n-fasted or non-fasted mice were anaesthetized using isoflurane and blood samples were taken as described above. Mice were kept at an unconscious stage using 1% isoflurane. M. gastrocnemius and M. soleus were excised rapidly, weighed and either
frozen in liquid nitrogen or stored in 4% neutral buffered formalin for subsequent preparation of paraffin blocks. Thereafter, mice were sacrificed by cervical dislocation and tissues were rapidly excised, weighed, and either frozen in liquid nitrogen or stored in formalin. Tissue weights were normalized to tibia length.

**In vivo nuclear magnetic resonance measurement of fat content**

Whole body fat mass was determined using a Minispec mq NMR analyzer (Brucker Optics, Woodlands, TX). Whole body fat mass is obtained as percentage of body weight and presented as gain or loss of fat mass compared to initial values.

**Magnetic Resonance Imaging (MRI)**

MR images were acquired at a 3T human MRI system (Siemens Tim-Trio, Erlangen, Germany) with an 8-channel multipurpose coil (Noras MRI products, Hoechenberg, Germany) to maximize signal-to-noise ratio. For fat-measurements a T1 weighted 2D Turbo Spin Echo (TSE) in coronal plane with the following parameters was used TR, 743 ms; TE, 39 ms; TF, 5. A field of view 90x45 mm with a 384x192 matrix was used to provide an in-plane resolution of 230x230 µm with a slice thickness of 0.9 mm. 24 excitations were averaged. For the final images a maximum intensity projection (MIP) was performed over all coronal slices with a self-developed software written in IDL (ITT Visual Information Solutions, Boulder, CO).

**Lipase activity assay (triacylglycerol hydrolase assay)**

Gonadal WAT was removed surgically as described above, rinsed in phosphate-buffered saline (PBS) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) using a Magna Lyser
The WAT lysate was centrifuged at 100,000 g for 1 h at 4 °C. The lipid-free infranatant (cytosolic fraction) was collected and used for TG hydrolase assays. The substrate for determining lipase activity contained non-labeled triolein and [9,10-3H(N)-triolein] (NEN Life Science Products, Boston, MA) as radioactive tracer that was emulsified with phosphatidylcholine/phosphatidylinositol using a conventional ultrasound sonicator. The cytosolic fractions with or without the specific HSL inhibitor 76-0079 (NNC 0076-0000-0079, Novo Nordisk, Bagsværd, Denmark) were incubated at 37 °C for 60 min under constant shaking. The reaction was terminated by the addition of methanol/chloroform/heptane (10:9:7), 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5). After centrifugation at 800 g for 20 min the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting in a LS 6500 Multi-Purpose Scintillation Counter from Beckman Coulter (Fullerton, CA).

**Ex vivo lipolysis of isolated WAT**

Gonadal fat pads were dissected and washed with PBS and cut into small pieces (~20 mg). The small WAT pieces were incubated in DMEM (PAA) containing 2 % fatty acid-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO) at 37°C. Aliquots of medium were analyzed for FA and glycerol content (release) by commercial kits (Wako Chemicals, Neuss, Germany and Sigma-Aldrich respectively).

**Proteasome assay**

*M. gastrocnemius* was homogenized in lysis buffer (20 mM Tris-HCl [pH 7.2], 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM DTT, 5 mM ATP, 20 % glycerol, 0.04 % (v/v) Triton X-100) using a magna lyser (Roche Diagnostics GmbH) at 4°C. The lysate was centrifuged at 13,000 g for 15 min at 4°C. The supernatant was collected and protein
concentration was determined using the RC DC Protein assay kit (BioRad, Hercules, CA). The chymotrypsin like proteasome activity was determined by incubating 40 µg protein with 0.167 µg/µl N-succinyl-Leu-Leu-Val-Try-7-amido-4-methylcoumarin (N-Suc LLVY-AMC, Sigma-Aldrich) in incubation buffer (100 mM Tris-HCl [pH 7.4], 50 mM HEPES [pH 8.0] and 5 mmol/l Ethyleneglycoltetraacetic-acid (EGTA)) for 60 min at 37°C. Fluorescence was determined using a conventional spectrofluorometer at 380 nm excitation and 460 nm emission.

Caspase activity assay

15 mg to 25 mg tissue from *M. gastrocnemius* was homogenized in 300 µl homogenization buffer (25 mM HEPES pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefabloc SC, 1 % protease-inhibitor cocktail [v/v] P8340 from Sigma-Aldrich) at 4°C. The lysate was centrifuged at 13,000 g for 20 min at 4°C. The supernatant was collected and the protein content was measured using the DC Protein Assay (BioRad). Caspase activity was determined by incubating 50 µg protein in 100 µl with 100 µl of Caspase-Glo 3/7 Reagent (Promega, Madison, WI, USA) for 1.5 h at 20°C and luminescence was determined using a luminometer (LUMIstar Optima BMG Labtec, Offenburg, Germany).

Blood and plasma parameters

Plasma glucose was measured using Accu-Check glucometer (Roche Diagnostics). Serum TG and FFA levels were determined using commercial kits from Thermo Electron, Thebarton, Australia and Wako Chemicals respectively.
Determination of TG content of cardiac muscle and *M. gastrocnemius*

Lipids were extracted from respective tissues by Folch extraction. The chloroform phase was evaporated using N₂ gas and the neutral lipids were solubilized in 2% Triton X-100 by sonication. TG was determined using Triglyceride FS kit (Diasys, Holzheim, Germany).

Determination of muscle and cardiac muscle protein content

*M. gastrocnemius* and heart samples were homogenized in RIPA lysis buffer (Fisher Scientific, Waltham, MA, USA) using a magna lyser (Roche Diagnostics GmbH) at 4°C. The lysate was centrifuged at 13,000 g for 15 min at 4°C. The supernatant was collected and protein concentration was determined using the DC Protein assay kit (BioRad).

IL-6 and TNF-α ELISA

Serum levels of TNF-α and IL-6 were measured using mouse TNF-α ELISA ReadySETGo and mouse IL-6 ELISA ReadySETGo (eBiosciences, San Diego, CA, USA). The plate was read at 450 nm using a conventional photometer.

Western blot analysis

Serum levels of zinc-α2-glycoprotein (ZAG) was determined by semi quantitative Western blot using ZAG (E-20) antibody (sc-11243, Santa Cruz, CA, USA) and consecutively the bands were analyzed densitometrically.

Determination of fatty acid transporter and fatty acid metabolizing gene mRNA

Total RNA from frozen sections of *M. gastrocnemius* was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. cDNA was
prepared from 1 µg of total RNA using high capacity reverse transcription kit (Applied Biosystems, Carlsbad, CA) and q-PCRs were performed using primers for CD36, Fatp-1, Cpt-1ß and Pgc-1α using Taqman Universal PCR master mix (Applied Biosystems) in triplicates. The relative expressions were calculated by the ddCt method using TF-2B as the housekeeping gene.

**Human adipose tissue samples**

Criteria for inclusion were malignant conditions. Nutritional status was assessed by body mass index (BMI). Patients were considered cachectic according to the definition of Evans et al. when BMI was < 20 and patients records indicated 3 or more of the additional criteria [decreased muscle strength, fatigue, anorexia, low fat-free mass index or abnormal biochemistry such as increased inflammatory markers (CRP, IL-6), anemia (Hb <12 g/dL) or low serum albumin (<3.2 g/dL)] (3). Non-tumor patients were used as control. Autopsies were performed within one day after demise. Samples of adipose tissue were collected from visceral adipose tissue and immediately snap frozen. The study had been approved by the ethics committee of the Medical University of Graz (20-096 ex 08/09).

**Statistical Methods**

Results are presented as mean ± standard deviation (S.D.). Comparisons were performed using unpaired t-test and are presented as *** for p ≤ 0.0001, ** for p ≤ 0.001, * for p ≤ 0.05.

For patient samples, non parametric Mann Whitney U test was performed and Spearman's rho was calculated using graphpad.
Figure legends

**Supplementary figure 1:**
Tumor weights after 14 d (A) and 21 d (B) post-injection of LLC in 12 h fasted lipase deficient mice were slightly lower than in Wt mice. n = 7

**Supplementary figure 2:**
Ablation of Atgl protects mice from cancer associated weight loss
Tumor development instigated significant weight loss in o/n fasted Wt and Hst⁻/⁻ mice 14 d (A) and 21 d (B) after injection of LLC tumor cells while Atgl⁻/⁻ mice retained weight despite tumor development. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. To allow direct comparison, values were determined after preparation and removal of the respective tumor. *** p<0.001, ** p<0.01, n = 7

**Supplementary figure 3:**
Changes in body weight are not attributable to dietary intake.
Food intake remained similar in LLC (A) and B16 melanoma (B) bearing mice compared to control mice of all genotypes. However, it tended to be reduce in the last 2-4 days of the experiment in all tumor bearing animals irrespective of genotype. n = 7

**Supplementary figure 4:**
Plasma glucose levels in tumor bearing and control Wt, Atgl⁻/⁻ and Hst⁻/⁻ mice.
(A, B) fasted glucose levels were reduced in tumor-bearing Wt and Hst⁻/⁻ mice compared to non-tumor-bearing littermates 21 d post tumor cell injection injection. This effect was not detected after 14 d. In contrast, plasma glucose levels remained unchanged in Atgl⁻/⁻ mice. (C, D) plasma glucose levels were not significantly changed
in non fasted LLC or B16 tumor-bearing mice compared to non-tumor-bearing littermates of any genotypes. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, n=7, except for (C), n = 3-5.

Supplementary figure 5:
Serum free fatty acid levels in tumor bearing and control Wt, Atgl\textsuperscript{−/−} and Hsl\textsuperscript{−/−} mice.

(A, B) fasted FA levels were increased in tumor-bearing Wt mice after 14 d and in tumor-bearing Hsl\textsuperscript{−/−} mice after 21 d. No changes were detected in Atgl\textsuperscript{−/−} mice. (C, D) Serum FA levels of non-fasted LLC and B16 tumor bearing Wt mice increased after 21 d and 16 d respectively. Atgl\textsuperscript{−/−} and Hsl\textsuperscript{−/−} mice did not show significant differences in serum FA levels between tumor and non tumor-bearing mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01,* p<0.05, n = 7 except for (C), n = 3-5.

Supplementary figure 6:
Serum TG levels in fasted tumor bearing and control Wt, Atgl\textsuperscript{−/−} and Hsl\textsuperscript{−/−} mice.

(A, B) fasted TG levels were reduced in Atgl\textsuperscript{−/−} and Hsl\textsuperscript{−/−} mice compared to Wt but were not significantly affected by tumor growth. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. n = 7

Supplementary figure 7:
Ablation of Atgl protects mice from cancer associated loss of adipose tissue.

(A, B, C) Total body fat content was significantly reduced in LLC and B16 tumor-bearing Wt and Hsl\textsuperscript{−/−} mice as determined by NMR. Total body fat content of tumor bearing Atgl\textsuperscript{−/−} mice, however, remained similar to non-tumor bearing Atgl\textsuperscript{−/−} mice. Black
Supplementary figure 8:
TG hydrolase activity is increased in WAT of LLC bearing Wt and Hsl<sup>−/-</sup> mice but not Atgl<sup>−/-</sup> mice. (A) total lipase activity, specifically inhibited (HSL, 76-0079) lipase activity (mainly ATGL activity), and HSL activity (determined by subtraction of inhibited lipase activity from total lipase activity) in Wt mice. Total lipase activity is increased mainly due to increased ATGL activity in tumor-bearing mice. (B) no significant change in lipase activity in Atgl<sup>−/-</sup> mice. (C) total (mainly ATGL) lipase activity is increased in Hsl<sup>−/-</sup> tumor-bearing mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, * p<0.05, n=7

Supplementary figure 9:
Total protein content of <i>M.gastrocnemius</i> is significantly reduced in B16 bearing Wt and Hsl<sup>−/-</sup> mice. No significant differences could be observed in Atgl<sup>−/-</sup> mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01, * p<0.05, n=7

Supplementary figure 10:
Effect tumor growth on slow twitch type I muscle (<i>M. soleus</i>) was less affected by (A, B) no significant change in <i>M. soleus</i> mass in LLC tumor bearing mice of any genotypes compared to saline injected mice after 14 d and 21 d. (C) <i>M. soleus</i> was significantly reduced in Wt B16 tumor bearing mice. Lipase deficient animals were protected from B16 tumor induced <i>M. soleus</i> loss. Muscle was dissected and its mass
normalized to *tibia* length as described in the methods section. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. * p<0.05, n=7

**Supplementary figure 11:**
Lipase deficient mice were protected from reduction of cardiac mass and cardiac protein. Heart weight tended to decrease in LLC (A) and B16 (B) bearing *Wt* mice compared to saline injected animals whereas *Atgl* or *Hsl* mice did not show significant differences. (C, D) Total protein content was reduced in LLC and B16 melanoma bearing *Wt* mice. Lipase deficient mice did not show any significant difference regardless of tumor development. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01,* p<0.05, n = 7

**Supplementary figure 12:**
Increase of TG levels in *M.gastrocnemius* and cardiac tissue of tumor bearing mice. (A) B16 melanoma bearing mice tended to have increased TG levels in *M. gastrocnemius*, the differences was, however, not significant. In *Wt* mice B16 (B) and LLC (C) tumors induced a significant TG increase in the heart, whereas heart TG levels remained unaffected in lipase deficient mice. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. *** p<0.001, ** p<0.01,* p<0.05, n = 7

**Supplementary figure 13:**
Changes of *M. gastrocnemius* were not yet observed 14 d after LLC tumor cell injection. (A) no change in muscle mass of *M. gastrocnemius* in tumor-bearing mice of all genotypes compared to saline injected mice after 14 d. (B) no change in
proteasome and (C) caspase activity in homogenates of M. gastrocnemius in tumorbearing mice of all genotypes. Black bars indicate control (saline injected) animals, white bars tumor-bearing animals. n=7

Supplementary Figure 14:

mRNA expression levels of FA metabolic genes were increased in M. gastrocnemius of tumor-bearing Wt and Hsl\(^{-/-}\) mice. mRNA expression of (A) Cd36, (B) Fatp1, (C) Cpt-1\(\beta\) and (D) Pgc-1\(\alpha\) determined by qRT-PCR was increased in tumor-bearing Wt and Hsl\(^{-/-}\) mice compared to control (saline injected) animals. No change was observed in the expression muscle of tumor-bearing Atgl\(^{-/-}\) mice. Black bars indicate control (saline injected) animals and white bars tumor-bearing animals. *** p<0.001, * p<0.05, n=7

Supplementary figure 15:

No significant correlation could be found between either (A) total lipase activity, (B) specifically inhibited (HSL, 76-0079) lipase activity (mainly ATGL activity), or (C) HSL activity (determined by subtraction of inhibited lipase activity from total lipase activity) in WAT and BMI of non-tumor patients.

Supplementary figure 1
Supplementary figure 2
Supplementary figure 3
Supplementary figure 4
Supplementary figure 5
Supplementary figure 6
Supplementary figure 7
Supplementary figure 8
Supplementary figure 9
Supplementary figure 10

(A) LLC (14 d)

(B) LLC (21 d)

(C) B16 (16 d)

Control

Tumor
Supplementary figure 11
Supplementary figure 12
Supplementary figure 13
Supplementary figure 14
Supplementary figure 15