Obesity attenuates inflammation, protein catabolism, dyslipidemia and muscle weakness during sepsis, independent of leptin

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SUPPLEMENTAL FILE

Supplemental methods

Animal study design

Male B6.V-Lep$^{ob/ob}$/JRj (Ob-KO) and male C57BL/6J mice were purchased at the age of 6 weeks (Janvier) and housed in a room with controlled temperature and 12:12 light-dark cycle. From 6 weeks of age, C57BL/6J mice were randomized to a high fat diet (60 KJ% fat, E15742-34, ssniff) or regular chow (9 KJ% fat, V1535-000) ad libitum for 11-12 weeks, generating diet-induced obese mice (Ob-DIO) and lean mice respectively. Leptin-deficient mice (Ob-KO) received a control diet (10 KJ% fat, E157453) ad libitum until they were 10 weeks old, thereafter they were calorie restricted to be weight and age matched with the Ob-DIO mice by week 17-18. One week before the cecal ligation and puncture (CLP) procedure, calorie restriction was omitted. During the course of their diet, obese mice (Ob-DIO and Ob-KO) were weighed on a weekly basis (additional figure 1a). At week 17-18 of age, lean mice were randomised to healthy control, septic placebo-treated mice (Ln) and septic leptin-treated mice (Ln-lep). Leptin treatment was started one hour before CLP, at a dose of 1mg/kg/d [1], divided in 2 doses per day for the remainder of the study (additional figure 1b). At the same age, Ob-DIO and Ob-KO obese mice were randomized to either healthy control or sepsis. Mice randomized to the sepsis group were anesthetized with ketamine and xylazine. A catheter was placed in the left central jugular vein, followed by 50% CLP with an 18G needle. After surgery, mice were fasted and received intravenous fluid resuscitation to mimic the clinical setting in which parenteral nutrition (PN) is initially withheld. After 24 hours, mice received standard mixed PN (5.8 kcal/day; Olimel N7E, Baxter). Throughout the study, mice received antibiotics (Imipenem/Cilastin) and analgesics (Buprenorphine). Pain/discomfort was assessed twice daily with the Mouse Grimace Score [2], and cumulative illness scores were calculated to assess illness severity. Mice randomized to healthy controls were individually caged receiving standard chow ad libitum. Mice were sacrificed 125h (5 days)

post-CLP. Sample size calculation was based on previous studies investigating the effect of obesity and muscle weakness in combination with the average mortality and technical failure rate of our sepsis model and aimed to reach at least 14 surviving animals per group (effect size of 1.11, α-error 0.05, power of 0.80). The Institutional Ethical Committee for Animal Experimentation of the KU Leuven had approved the protocol (internal project number P181-2016).

Body composition analysis and ex vivo muscle force measurements

Body composition was measured immediately before CLP and immediately before sacrifice, at 125h post CLP, with magnetic resonance imaging (echoMRI-100H, Whole Body Magnetic Resonance Analyser, Zinsser Analytic GmbH). Directly after euthanasia, the hindlimb m. extensor digitorum longus (EDL) was carefully dissected and suspended in a temperature controlled (30°C) organ bath filled with HEPES-fortified Krebs-Ringer solution to measure muscle force (300C-LR Dual-Mode muscle lever, Aurora Scientific) [3]. The small size of the EDL guaranteed proper diffusion of oxygen during the procedure. Maximal isometric tetanic force was measured by averaging three consecutive tetanic stimuli (180 Hz stimulation frequency, 200 ms duration, 0.2 ms pulse width, 2 min rest intervals). Specific maximal isometric tetanic force was calculated by dividing the maximal isometric tetanic force with the muscle cross-sectional area.

Whole blood and plasma analyses

Blood glucose and ketone concentrations were measured on whole blood drawn from the tail vein with Accu-Check (Roche) and StatStrip Xpress 2 (Nova Biomedical) respectively. Plasma urea nitrogen (EIABUN, Invitrogen), free fatty acids (7010310, Cayman Chemical

Company), glycerol (MAK117, Sigma-Aldrich) LDL and HDL cholesterol (DZ128A-K and DZ129A-K, Diazyme Laboratories) TNFα (MHSTA50, R&DSystems), IL-6 (M6000B, R&DSystems, corticosterone (EIA5186, DRG) and leptin (EZML82K, Millipore) were measured using commercial assays.

Gene and protein analyses

RNA was isolated with Qiazol and the RNeasy mini RNA isolation kit (QIAGEN). DNAse treatment removed genomic DNA. RNA was reverse-transcribed with the use of random hexamers. Relative gene expression was determined with the 2-ΔΔCt method with 18S ribosomal RNA (Rn18s) as housekeeping gene (Applied Biosystems). An overview of the gene expression assays used for markers of muscle wasting and weakness, inflammation and lipid metabolism is provided in Additional table 1. For protein analysis, tissue samples were homogenized in a buffer containing 20 mM Tris-HCl, pH 7.6, 10% glycerol, 1% Nonidet P-40, 2 µg/ml aprotinin, 5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 10 mM sodium orthovanadate, 34 µg/ml phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 10 mM EDTA. The protein content was determined with Coomassie Protein Assay Reagent (Pierce Biotechnology Inc.) using a standard curve of BSA. Western blots were performed using commercial 4-20% tris-glycine gels (Biorad) and PVDF membranes (Thermo Fisher scientific). Blots were incubated with primary antibodies for markers of autophagy LC3B (L7543, Sigma Aldrich) and p62 (H00008878-M01, Novus Biologicals), and protein synthesis p70 S6 kinase (#9202 and #9205, Cell Signaling) and corresponding secondary horseradish peroxidase-conjugated antibodies (DakoCytomation). Blots were developed with the Western Lightning chemiluminescence reagent Plus kit (Perkin Elmer), visualized with the G:BOX Chemi XRQ (SynGene) and analyzed with the SynGene software.

Histology

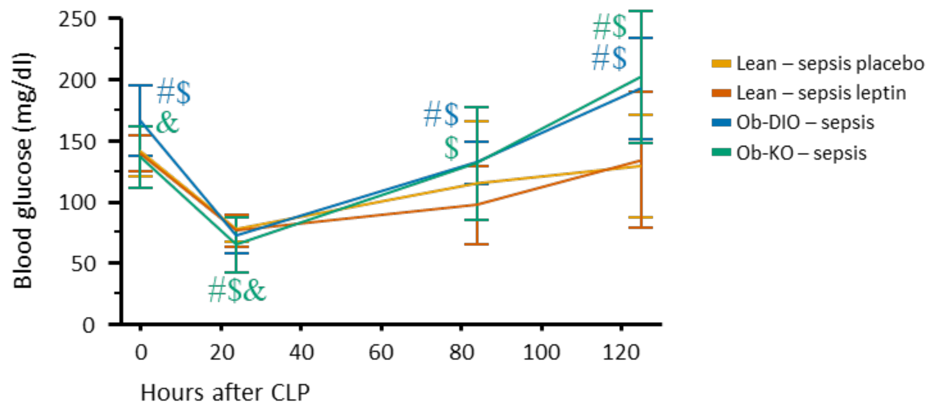
The right tibialis anterior muscle and a liver sample were fixed with paraformaldehyde and embedded in paraffin. Paraffin embedded sections were stained with Haematoxylin & Eosin and scored semi-quantitatively. Liver sections were scored on inflammation, vacuolisation, sinusoidal dilation, and fat content. All parameters were classified as either absent, mild or moderate, except for fat content which scoring was based on the percentage of fat droplets in the liver section (<5%; 5-33%, 33-66%, >66%). Muscle sections were scored on inflammation, fibrosis, necrosis, adipocyte infiltration, presence of angular fibers and presence of centrally nucleated fibers. All parameters were classified as either absent, mild or moderate, except for necrosis which was scored as present if at least two of three following parameters were present on more than half of the investigated slides (myophagocytosis, hypereosinophily, pale myofibers). For muscle fiber type staining, cryosections of the left tibialis anterior muscle were blocked with mouse-on-mouse (MOM) blocking reagent and incubated overnight at 4°C with primary antibodies; BA-F8-s, SC-71-s, BF-F3-c (Developmental Studies Hybridoma Bank, Iowa, USA), ab11575 (Abcam, Cambridge, UK). After washing with PBS, cryosections were incubated with the conjugated secondary antibodies: IgG2b-AF350, IgG1-Cy5, IgM-AF594, goat anti-rabbit-AF488 (Thermo Fisher scientific, Waltham, MA). Whole slides were scanned with TissueFAXS i PLUS microscope (TissueGnostics, Vienna, Austria) and analysed with MuscleJ software [4].

Supplemental table 1: list of commercially used gene expression assays (Applied Biosystems)

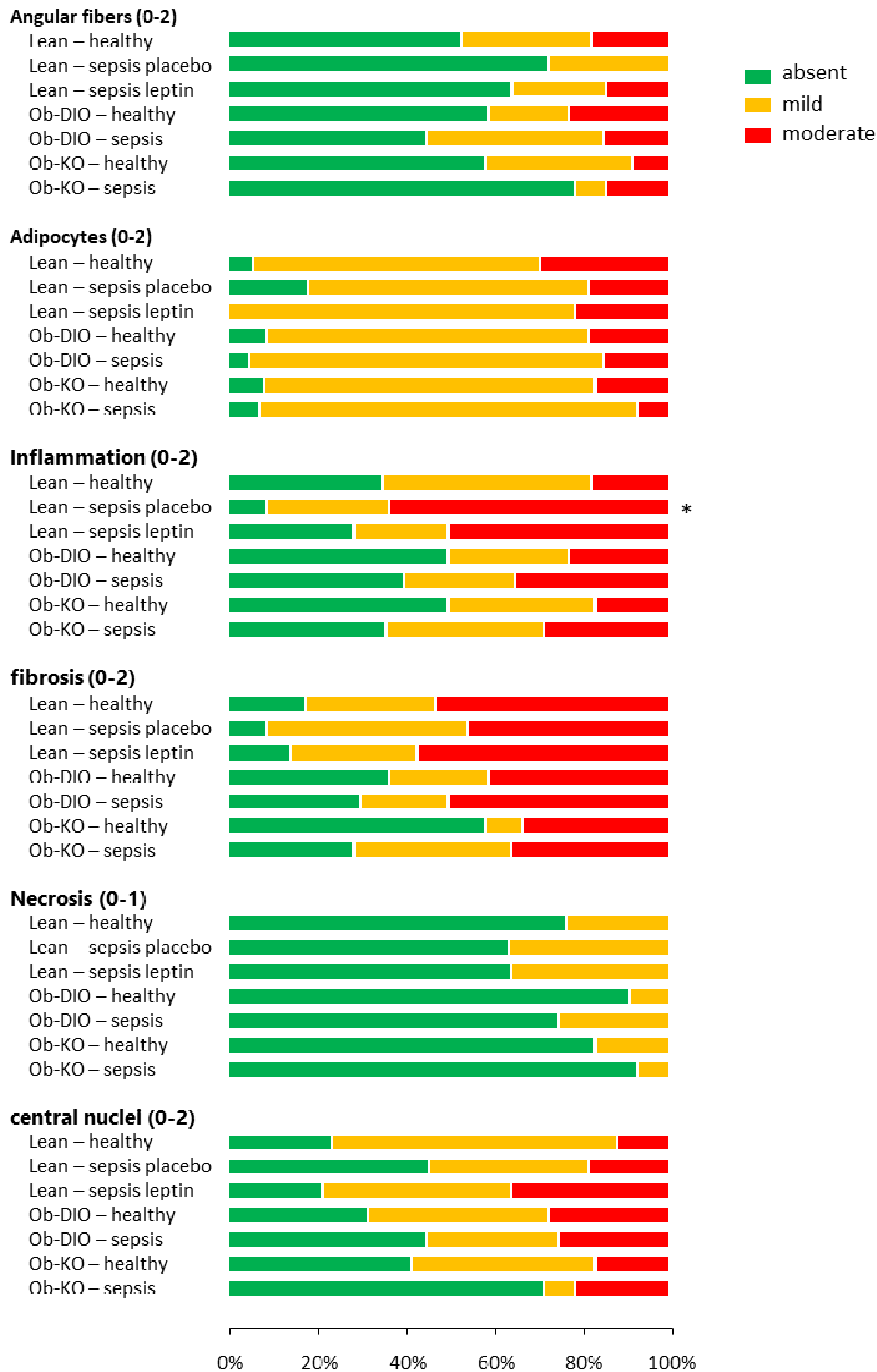
Aass	Mm01165010_m1
Acadl	Mm00599660_m1
Acta1	Mm008008218_g1
Arg1	Mm00475988_m1
Cpt1α	Mm01231183_m1
Fbxo32	Mm00499523_m1
Got1	Mm01195792_g1
Hdac4	Mm01299557_m1
Hdac5	Mm01246076_m1
Il-1β	Mm00443258_m1
Il-6	Mm00446190_m1
Mef2c	Mm01340842_m1
Mstn	Mm01254559_m1
Myf5	Mm00435125_m1
Myh1 (IIx)	Mm01332489_m1
Myh2 (IIa)	Mm01332564_m1
Myh4 (IIb)	Mm01332516_g1
Myh7 (I)	Mm00600555_m1
Myod1	Mm00440387_m1
Myog	Mm00446194_m1
Nlrp3	Mm00840904_m1
Oat	Mm00497544_m1
Pcna	Mm00448100_g1
Rn18s	Mm03928990_g1
Sds	Mm00455131_m1
Tnfα	Mm00443258_m1
Trim63	Mm01185221_m1
Ubb	Mm01622233_g1
Ubc	Mm01198158_m1

Supplemental table 2: baseline characteristics of lean and overweight/obese ICU patients

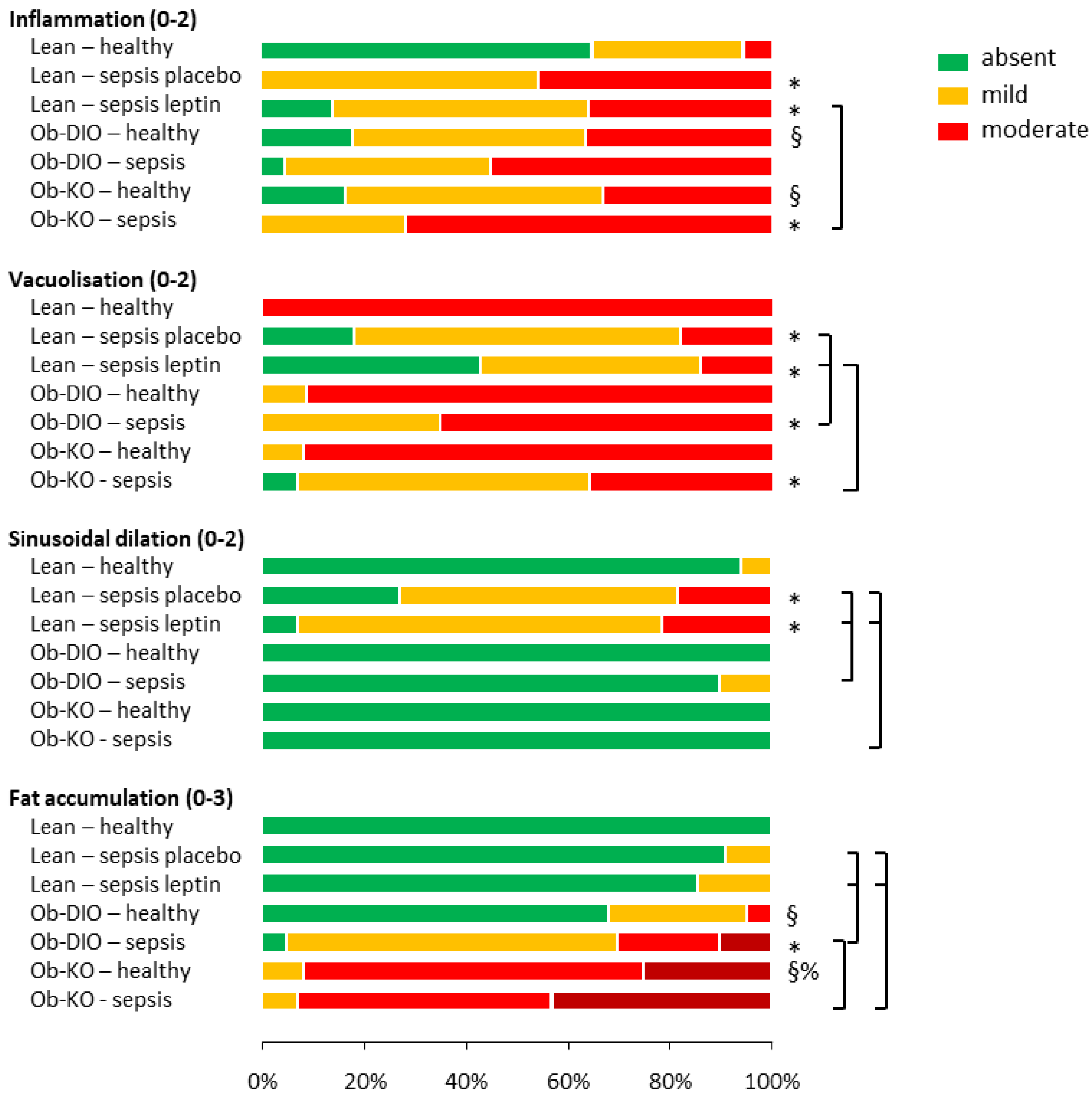
BASELINE CHARACTERISTICS	LEAN (N=694)	OVERWEIGHT/OBESE (N=694)	STANDARDIZED DIFFERENCE OF MEAN [5]
BMI (kg/m²) – median (IQR)	22.5 (20.7-23.8)	28.6 (26.7-31.2)	
Propensity score			0.098
Age (years) - median (IQR)	63 (50-74)	69 (57-76)	0.426
Sex (male) - no. (%)	425 (61)	417 (60)	0.020
Randomization to late PN- no. (%)	345 (50)	323 (47)	0.060
Diagnostic group – no. (%)			
Surgical			
Emergency – no. (%)	381 (55)	325 (47)	0.160
Cardiac surgery – no. (%)	264 (38)	333 (48)	0.203
Other surgery– no. (%)	44 (6)	34 (5)	0.043
Medical – no. (%)	5 (1)	2 (1)	0.000
History of malignancy – no. (%)	260 (20)	316 (16)	0.104
APACHE-II score – median (IQR)	30 (21-36)	28 (19-36)	0.284
OUTCOME			
ICU mortality – no. (%)	65 (9)	80 (12)	
Length of ICU stay (days) – median (IQR)	10 (6-19)	11 (5-21)	



Supplemental Figure 1: Blood glucose. Blood glucose concentration. # $p<0.05$ compared to lean septic placebo treated mice; $ $p<0.05$ compared to lean septic leptin treated mice; & $p<0.05$ compared to septic Ob-DIO mice. The colour of the mark refers to the comparator.



Supplemental Figure 2: semi-quantitative scoring of tibialis anterior H&E sections. Data are shown as cumulative percentages of the respective group. Number (n) of animals: Lean healthy n=17; lean sepsis placebo n=11, lean sepsis leptin n=14, Ob-DIO healthy n=22, Ob-DIO sepsis n=20, Ob-KO healthy n=12, Ob-KO sepsis n=14. * $p<0.05$ between septic mice and healthy controls.



Supplemental Figure 3: semi-quantitative scoring of liver H&E sections. Data are shown as cumulative percentages of the respective group. Number (n) of animals: Lean healthy n=17; lean sepsis placebo n=11, lean sepsis leptin n=14, Ob-DIO healthy n=22, Ob-DIO sepsis n=20, Ob-KO healthy n=12, Ob-KO sepsis n=14. * $p<0.05$ between septic mice and healthy controls; § $p<0.05$ compared to lean healthy mice; % $p<0.05$ compared to healthy Ob-DIO mice; brackets between groups indicated $p<0.05$.

References

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