

1 **TRAIL reduces impaired glucose tolerance and NAFLD in the high-fat diet-fed**
2 **mouse**

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23 **Abstract**

24 Aims/hypothesis: Recent studies suggest that a circulating protein called TRAIL (TNF-related
25 apoptosis-inducing ligand) may have an important role in the treatment of type 2 diabetes. It has been
26 shown that TRAIL deficiency worsens diabetes and that TRAIL delivery, when it is given before
27 disease onset, slows down its development. This study aimed at evaluating whether TRAIL had the
28 potential not only to prevent, but also to treat type 2 diabetes.

29 Methods: Thirty male C57BL/6J mice were randomized to a standard or a high-fat diet (HFD). After 4
30 weeks of HFD, mice were further randomized to receive either placebo or TRAIL, which was delivered
31 weekly for 8 weeks. Body weight, food intake, fasting glucose and insulin were measured at baseline
32 and every 4 weeks. Tolerance tests were performed before drug randomization and at the end of the
33 study. Tissues were collected for further analyses. Parallel *in vitro* studies were conducted on HepG2
34 cells and mouse primary hepatocytes.

35 Results: TRAIL significantly reduced body weight, adipocyte hypertrophy, free fatty acid levels and
36 inflammation. Moreover, it significantly improved impaired glucose tolerance, and ameliorated non-
37 alcoholic fatty liver disease (NAFLD). TRAIL treatment reduced liver fat content by 47% *in vivo* as
38 well as by 45% in HepG2 cells and by 39% in primary hepatocytes. This was associated with a
39 significant increase of liver PGC-1 α expression both *in vivo* and *in vitro*, pointing to a direct protective
40 effect of TRAIL on the liver.

41 Conclusion/interpretation: This study confirms the ability of TRAIL to attenuate significantly diet-
42 induced metabolic abnormalities, and it shows for the first time that TRAIL is effective also when
43 administered after disease onset. In addition, our data sheds light on TRAIL therapeutic potential not
44 only against impaired glucose tolerance, but also against NAFLD.

45 **Keywords:** high-fat diet, impaired glucose tolerance, type 2 diabetes, NAFLD, NASH, mouse model,
46 TRAIL, PPAR γ , PGC-1 α .

47 **Abbreviation list**

48 AOX, Acyl-CoA oxidase; ATG7, autophagy-related protein 7; CPT carnitine palmitoyl transferase;
49 CPT1a, carnitine palmitoyl transferase-1a; CRP, C-reactive protein; CYT SYNT, cytrate synthase; FAS
50 fatty acid synthase; gp91phox is a subunit of NADPH oxidase; HFD, high-fat diet; IPITT,
51 intraperitoneal insulin tolerance test; HNF4, hepatocyte nuclear factor 4; MCP1, monocyte
52 chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic
53 steatohepatitis; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 alpha; pgWAT,
54 perigonadal white adipose tissue; PLN5, perilipin 5; PPAR α , peroxisome proliferator-activated
55 receptor- α ; PPAR γ , peroxisome proliferator-activated receptor- γ .; SIRT1, sirtuin-1; SREBP1a, sterol
56 regulatory element binding protein-1a; SREBP1c, sterol regulatory element binding protein-1c;
57 TRAIL, TNF-related apoptosis-inducing ligand; UCP2, uncoupling protein.

58

59 **Introduction**

60 TRAIL is an acronym for TNF-related apoptosis-inducing ligand, which is a circulating protein
61 belonging to the TNF superfamily. Like other members of this family, such as FasL and TNF α , TRAIL
62 has the ability to induce programmed cell death (apoptosis). However, as compared to them, TRAIL
63 hits preferentially transformed cells, such as cancer cells, while sparing the normal ones (1). In non-
64 malignant cells, TRAIL actions remain largely unexplored.

65 Recent experimental evidence suggests that TRAIL has significant metabolic effects (2, 3), and might
66 be involved in the regulation of obesity and diabetes mellitus, as well as their complications. The first
67 studies reporting TRAIL beneficial effects on diabetes were carried out in models of type 1 diabetes
68 and showed that TRAIL attenuated disease development and progression (4, 5) with partial
69 preservation of islet morphology (6). More recently, we reported that TRAIL delivery significantly
70 reduced the metabolic abnormalities of an experimental model of type 2 diabetes (7). Consistent with
71 these observations, other groups have shown that genetic lack of TRAIL worsened both forms of
72 diabetes and their associated diseases (8), such as atherosclerosis (8) and non-alcoholic fatty liver
73 disease (NAFLD) (9).

74 The high-fat diet (HFD)-fed mouse is one of the models suitable for the study of type 2 diabetes (10).
75 After a few weeks, the HFD usually increases body weight and fat mass, leading to peripheral insulin
76 resistance and impaired glucose tolerance, with subsequent hyperglycemia and hyperinsulinemia (10).
77 Although the HFD-fed mouse does not always develops diabetes, but rather impaired glucose
78 tolerance, it has the advantage of reproducing the human situation more accurately than genetic models
79 of obesity-induced diabetes (11). Moreover, it allows for the study of common and burdensome
80 diabetic comorbidities, such as obesity, NAFLD, and non-alcoholic steatohepatitis (NASH) (12).

81 A few years ago, we reported that TRAIL significantly ameliorated the metabolic abnormalities of the
82 HFD-fed mouse. In that paper, TRAIL treatment was started before the development of metabolic
83 abnormalities, following a preventive treatment schedule. In this study, we aimed at evaluating the
84 therapeutic potential of TRAIL against type 2 diabetes. For this reason, TRAIL treatment was started
85 after the development of metabolic abnormalities, following a therapeutic schedule. Here we report the
86 effects of TRAIL treatment on glucose tolerance and on the tissues regulating it.

87

88

89 **Materials and Methods**

90 **Recombinant human TRAIL (rhTRAIL)**

91 Recombinant histidine 6-tagged human TRAIL (114-281) was produced in transforming bacteria BL21
92 with a pTrc-His6 TRAIL vector, as described (13) and detailed in the **Supplementary Material and**
93 **Methods**.

94 **Experimental protocol**

95 As reported in **Figure 1A**, thirty 8-week-old C57BL/6J male mice (Harlan Laboratories, Udine, Italy)
96 were randomized to receive either a standard diet (CNT, n=10) or a high-fat diet (HFD, n=20) for 12
97 weeks. After 4 weeks from diet randomization, HFD mice were randomly allocated to receive either
98 saline (HFD, n=10) or rhTRAIL (HFD + TRAIL, n=10) for the remaining 8 weeks. CNT mice received
99 saline (NaCl 0.9%) as well. rhTRAIL (TRAIL) was given at a dose of 10 µg/200µL per mouse by
100 weekly intraperitoneal (IP) injection. Standard diet provided 22% of calories from protein, 66% of
101 calories from carbohydrate, and 12% of calories from fat, and a digestible energy of 3.0 Kcal/g
102 (Tekland Global 16% Protein Rodent Diet, Harlan Laboratories). HFD provided 18.4% of calories from
103 protein, 21.3% of calories from carbohydrate, and 60% of calories from fat, and a digestible energy of
104 5.1 Kcal/g (TD 06.414 Adjusted calories diet 60/fat, Harlan Laboratories). Animals were kept (5/cage)
105 in ventilated cabinets (Tecniplast Spa, Buggiate, Varese, Italy), in specific-pathogen-free and
106 temperature-controlled rooms (22°C), with relative humidity of 50-70%, on a 12h light/12h dark cycle.
107 They had free access to food and water, and they were fed *ad libitum* for the length of the study. During
108 the 12-week study period, body weight, food intake, glucose, and insulin were measured at baseline
109 and at 4-week intervals. Tolerance tests were performed before drug randomization and at the end of
110 the study. At the end of the study, animals were anesthetized by an IP injection of tiletamine/zolazepam

111 (80mg/kg). Blood was collected from the left ventricle, centrifuged, and serum was stored for further
112 analyses (total cholesterol, HDL cholesterol, triglycerides, free fatty acids, C-reactive protein).
113 Pancreas, perigonadal white adipose tissue (pgWAT), liver, and quadriceps were weighed and either
114 snap frozen or fixed in formalin for further analyses.

115 The Guide for the Care and Use of Laboratory Animals, 8th edition (2011), as well as specific
116 European (86/609/EEC) and Italian (D.L.116/92) laws were followed. In compliance with the principle
117 of reducing as much as possible the number of mice studied, we did not include CNT mice + TRAIL
118 because we have previously observed and reported that repeated injections of TRAIL (providing a
119 cumulative dose of 100 µg/mouse) did not affect glucose, insulin, and/or body weight in normal
120 conditions *in vivo* (6). This study was approved by the Institutional Animal Care and Use Committee of
121 the Cluster in Biomedicine (CBM) and by the Italian Ministry of Health (DM 17/2001 A dd.
122 02/02/2011). The study period was June 2015-May 2017.

123 **Assessment of TRAIL biodistribution**

124 In order to evaluate TRAIL bioavailability when delivered by IP injection, 20-week-old C57BL/6J
125 male mice (n=3) received 10 µg of Cy5.5-TRAIL by IP injection, and fluorescence in the peritoneal
126 cavity was acquired at baseline and after 30 minutes, 6 hours, and 30 hours post-injection, as
127 previously reported (14). The details of TRAIL labeling are in the **Supplementary Materials and**
128 **Methods**. In addition, 20-week-old C57BL/6J male mice (n=8) received 10 µg of TRAIL by IP
129 injection, bloods were collected at baseline and after 6, 30, 48, and 72 hours, and TRAIL was measured
130 by ELISA (R&D; #DTLR00).

131 **General parameters and biochemistries**

132 Food intake was measured by placing in the cages pellets previously weighed in total. The food that
133 was left over was collected and weighed to find the amount eaten. Energy intake was measured
134 according to the digestible energy provided by both diets. Fasting glucose was measured by glucometer
135 (Glucomen LX Plus, Menarini). Fasting insulin was measured by ELISA (Millipore; #EZRMI-13K).
136 NEFA concentrations were measured by colorimetric assay (Sigma; #MAK044-1KT). Circulating total
137 cholesterol, HDL cholesterol, and triglycerides were measured with the AU5800 analyzer (Beckman
138 Coulter) by enzymatic colorimetric method, while C-reactive protein (CRP) was measured by
139 immunoturbidimetric method.

140 **Tolerance tests**

141 The intraperitoneal glucose tolerance test (IPGTT) was performed on day 1 of week 4 and week 12 by
142 injecting glucose (2 g/kg) intraperitoneally after an overnight fast. Glucose and insulin were measured
143 at baseline and at 15, 60, and 120 min. The intraperitoneal insulin tolerance test (IPITT) was performed
144 on day 1 of week 3 and week 11 by injecting insulin (1 unit/kg) intraperitoneally after a 6-hour fast.
145 Glucose was measured at baseline, and at 30, 60, and 120 min.

146 **Tissue Stainings**

147 Adipocyte area was measured on pgWAT paraffin sections (4 μ m) stained with H&E. pgWAT
148 macrophages were detected by F4/80 immunostaining (1:100 dilution, applied overnight; Abcam
149 #Ab111101) and reported as positive nuclei/100 nuclei. Pancreatic beta cell density and mass were
150 estimated as previously described (15) on paraffin sections (4 μ m) by insulin immunostaining (1:100
151 applied overnight; DAKO #A0564). Liver steatosis was evaluated on frozen sections (5 μ m) stained
152 with Oil-Red-O, where fat was quantified as % of positive (red) staining/tissue area. The same method
153 was used for skeletal muscle fat content. For liver fibrosis, paraffin sections (4 μ m) were stained with

154 Picrosirius Red, and fibrosis was quantified as % of positive (red) staining/tissue area (15). Liver
155 macrophages were detected on frozen sections (5 μ m) by CD68 immunostaining (1:50 applied
156 overnight; Serotec #MCA1957S), and they were reported as positive nuclei/frame. Liver PGC-1 α
157 expression was measured on paraffin sections (4 μ m) by PGC-1 α immunostaining (1:50 applied
158 overnight; Abcam #ab191838) and was reported as the % of positive (brown) staining/tissue area. All
159 the sections were examined by light microscopy (Carl Zeiss - Jena) and digitized using a high-
160 resolution camera (Q-Imaging Fast 1394). The % of staining/tissue area was quantified using Image-
161 ProPlus 6.3 (Media Cybernetics, Bethesda, MD, USA). Quantifications were performed on 40-100
162 frames per group.

163 **Liver triglyceride content**

164 For liver triglycerides (TG), 100 mg of liver were homogenized in 1 ml of 5% NP40. Samples were
165 heated at 95° for 5 minutes, cooled down at RT for 5 minutes twice, and then centrifuged to collect the
166 supernatants. Triglycerides were measured with the AU5800 analyzer (Beckman Coulter) by enzymatic
167 colorimetric method (for details, see the **Supplementary Materials and Methods**).

168 **Gene expression quantification by RT-qPCR**

169 Gene expression was determined by real-time quantitative RT-qPCR. In order to isolate mRNA, tissue
170 was homogenized and processed as previously reported (16). Then, mRNA was treated to eliminate
171 DNA contamination (Ambion DNA-free product #AM-1906), and 3 μ g of treated mRNA were
172 subsequently used to synthesize cDNA with Superscript First Strand synthesis system for RT-PCR
173 (Gibco BRL). The gene expression of *Fas*, *Srebp1a*, *Srebp1c*, *Acox*, *Cpt1a*, *Ppara*, *Irs2*, *Pepck*, *Ppar γ* ,
174 *Hnf4*, *Citrate synthase*, *Pgc-1 α* , *Ucp2*, *Sirt*, *Pln*, *Gp91phox*, *Il-6*, *Tnfa*, and *Atg7* was analyzed by RT-
175 qPCR using the SYBR Green system (Life Technologies). *Mcp1* was analyzed using the Taqman

176 system (Life Technologies). Fluorescence for each cycle was quantitatively analyzed by StepOnePlus
177 real-time PCR system (Applied Biosystems). Gene expression was normalized to *Rps9* or *18s*. Results
178 are reported as ratio compared with the level of expression in untreated controls, which were given an
179 arbitrary value of 1. Primers are reported in **Supplementary Table 1**.

180 ***In vitro* studies on HepG2 cells and mouse primary hepatocytes**

181 Human hepatocellular carcinoma cells (HepG2) obtained from ATCC were cultured in high glucose
182 DMEM, supplemented with 10% (v/v) fetal bovine serum, L-glutamine (2mM), penicillin (100U/mL),
183 and streptomycin (100 µg/mL) in a 5% CO₂ atmosphere at 37°C. In order to mimic a high-fat diet
184 *milieu*, cells were grown in media supplemented with either BSA-conjugated palmitate (250 mM) or
185 BSA-conjugated oleate (250 mM). Cells were cultured for 24 hours with BSA-conjugated oleate with
186 or without TRAIL (1 ng/mL) for cell viability, lipid accumulation, and protein expression studies,
187 while they were cultured for 6 hours for gene expression analysis. Cell viability was assessed with the
188 MTT assay (Alfa Aesar; #L11939). Total intracellular lipid content was evaluated by Oil-Red-O
189 staining. Protein expression of PGC-1 α was analyzed by immunofluorescence. Gene expression of
190 *PPAR γ* , *PGC-1 α* , and *ATG7* was analyzed by RT-qPCR using the SYBR Green system (Life
191 Technologies). cDNA was synthesized as detailed for our *in vivo* analyses. Fluorescence for each cycle
192 was quantitatively analyzed by StepOnePlus real-time PCR system (Applied Biosystems). Gene
193 expression was normalized to GAPDH or Rpl27, and reported as a ratio compared to the level of
194 expression in untreated controls, which were given an arbitrary value of 1. Primers are reported in
195 **Supplementary Table 1**.

196 In addition, in parallel *in vitro* studies, mouse primary hepatocytes were isolated from C57BL/6J male
197 mice following a protocol adapted from Severgnini (17). After digestion with collagenase IV, liver
198 suspension was passed through a cell strainer. Primary hepatocytes were washed 4 times and seeded on

199 pre-coated plates with a plating medium (Williams E Medium supplemented with CM3000,
200 ThermoFisher Scientific) for 5 hours in a 5% CO₂ atmosphere at 37°C. Then, this medium was
201 changed to a maintenance solution (CM4000 ThermoFisher Scientific) for 24 hours before starting the
202 treatment. As before, primary hepatocytes were cultured for 24 hours with BSA-conjugated oleate
203 (250mM) or BSA alone, with or without TRAIL (1 ng/mL). Total intracellular lipid content was
204 evaluated by Oil-Red-O staining.

205

206 **Statistics**

207 Results are expressed as means ± standard error of the mean (SEM). Differences in the mean among
208 groups were analyzed using one-way or two-way ANOVA. Pairwise multiple comparisons were made
209 using Bonferroni post-hoc analysis. A threshold of $p < 0.05$ was considered statistically significant.

210 **Results**

211 **Animal model and *in vivo* bioavailability of TRAIL**

212 Mice were put on a high-fat dietary regime to induce obesity and associated metabolic comorbidities
213 (**Figure 1A**). After 4 weeks, before drug randomization, HFD mice had increased in weight (6.3 ± 0.6
214 g) as compared to CNT mice (3.7 ± 0.6 g; $p < 0.05$ vs HFD). HFD mice weighed 30.4 ± 0.5 g and CNT
215 mice weighed 28.9 ± 0.7 g. There were no differences between the groups in terms of fasting glucose
216 and insulin. Nevertheless, the glucose and insulin tolerance tests showed that HFD mice had developed
217 impaired glucose tolerance as compared to CNT mice (**Figure 1B**). At this stage, HFD mice were
218 randomized to receive either TRAIL or saline by weekly IP injection for 8 weeks. This schedule was
219 chosen based on our previous study (7). Mice tolerated this treatment well, and they did not show any
220 sign of distress during the study or gross abnormalities at necroscopic examination as compared to
221 those treated with saline, in line with previous reports (18). When we then looked at TRAIL
222 bioavailability, we found that TRAIL was detectable after 6 hours from the IP injection and then
223 disappeared 24 hours later. This data was in line with our imaging experiment showing that the highest
224 fluorescence signal of Cy5.5-TRAIL was detected 6 hours after the IP injection, and then tended to
225 decrease over time (**Figure 1C-D**).

226 **TRAIL treatment significantly reduced obesity, as well as adipose and systemic inflammation**

227 At the end of the study, HFD mice became obese as compared to CNT mice. TRAIL treatment
228 significantly reduced body weight gain in HFD mice (**Figure 2A**), without affecting their food and
229 energy intake. In particular, HFD mice ate 2.7 ± 0.2 g/day with an energy intake of 13.7 ± 1.2
230 Kcal/day, as compared to HFD+TRAIL mice, who ate 2.6 ± 0.3 g/day with an energy intake of $13.4 \pm$
231 1.3 Kcal/day. Secondly, HFD mice exhibited WAT hypertrophy, as assessed by pgWAT weight

232 (**Figure 2B**), which is considered one of the largest visceral WAT depots (19). This was associated
233 with an increase in the adipocyte area (**Figure 2C-E**) and the number of infiltrating macrophages
234 (**Figure 2E-F**). TRAIL treatment significantly reduced WAT hypertrophy, adipocyte area, and
235 macrophage accumulation in the WAT (**Figure 2B-F**). Thirdly, HFD mice exhibited higher levels of
236 NEFA and CRP (**Figure 2G-I**), in line with the concept that excess fat is associated with high levels of
237 NEFA (20) and a low-grade systemic inflammatory state (21). TRAIL treatment significantly reduced
238 both NEFA and CRP (**Figure 2G-I**). Interestingly, TRAIL treatment was associated with a significant
239 increase in total cholesterol, driven by an increase in HDL cholesterol.

240 **TRAIL treatment significantly reduced impaired glucose tolerance.**

241 The tolerance tests (**Figure 3A-F**) performed at the end of the study showed that the HFD resulted in
242 impairment of glucose clearance, leading to significant hyperglycemia and hyperinsulinemia 2 hours
243 after a glucose load (**Figure 3D-E**). This was associated with the inability of insulin to lower glucose
244 levels to the levels of CNT mice (**Figure 3F**). At fasting, glucose did not differ between the groups,
245 and HFD mice displayed only a significant hyperinsulinemia as compared to CNT mice (**Figure 3G**),
246 indicating the presence of an insulin resistance with impaired glucose tolerance. This data was
247 consistent with the lack of significant changes in β -cell mass and density (**Figure 3H; Supplementary**
248 **Figure 1**). TRAIL treatment significantly reduced both impaired glucose tolerance and insulin
249 resistance (**Figure 3A-G**).

250 **TRAIL treatment significantly reduced NAFLD**

251 To understand why TRAIL reduced impaired glucose tolerance, we looked at the tissues regulating
252 glucose metabolism, focusing on the liver and skeletal muscle (22). At the end of the study, the HFD
253 regime did not increase the deposition of fat (lipid droplets) in the skeletal muscle, as assessed by Oil-

254 Red-O staining (data not shown). By contrast, when we looked at the liver, HFD mice developed
255 significant steatosis (lipid accumulation >10% of tissue area), which was assessed by Oil-Red-O
256 staining and by TG content quantification (**Figure 4 A-C**). The accumulation of more than 5-10% of
257 fat in the liver, without any primary cause such as viral hepatitis, alcoholic disease, or drug-induced
258 liver injury is what defines histologically non-alcoholic fatty liver disease (NAFLD) (23). In our study,
259 HFD mice also exhibited a significant increase in liver macrophage infiltration (**Figure 4C-D**), but no
260 fibrosis, which should appear after about 50 weeks of HFD (12). TRAIL treatment significantly
261 reduced liver steatosis as well as inflammation, therefore ameliorating HFD-induced NAFLD.

262 **TRAIL significantly increased PGC-1 α expression in the liver**

263 In parallel studies, we quantitated liver mRNA encoding transcription factors and metabolic enzymes
264 involved in *de novo* lipogenesis, fatty acid oxidation, glucose metabolism, and mitochondrial function,
265 as well as pro-oxidative and proinflammatory molecules. In the liver, HFD significantly increased the
266 gene expression of *Fas*, *Ppar γ* , and *Il-6*, while it decreased that of *Cpt1 α* , *Pepck*, and *Pgc-1 α* , as
267 compared to CNT mice (**Table 1**). In HFD mice, TRAIL significantly decreased the gene expression of
268 *Fas* and *Il-6*, while it increased that of *Ppar γ* and *Pgc-1 α* (**Table 1; Figure 4E-F**). The increase in
269 *Ppar γ* and *Pgc-1 α* gene expression that followed TRAIL treatment was observed only in the liver
270 (**Supplementary Figure 2A**). Given that PGC-1 α regulates energy metabolism and could explain part
271 of our findings, we further evaluated it by immunostaining. In HFD mice, PGC-1 α protein expression
272 increased and changed pattern of distribution as compared to CNT mice, where it was located in the
273 nuclei. TRAIL further increased PGC-1 α protein expression in the liver (**Figure 4C and Figure 4G**).
274 In addition, we quantitated liver mRNA encoding for *Atg7*, which is a protein essential for autophagy, a
275 process that has been recently implicated in lipid metabolism regulation (24). Interestingly, an increase
276 in *Atg7* was observed in the liver of HFD+TRAIL mice (**Table 1; Supplementary Figure 2A**)

277 **TRAIL significantly reduced lipid droplet accumulation in hepatocytes cultured in a high-fat diet**
278 *milieu*

279 To determine whether TRAIL had direct effects on hepatocytes, we used an *in vitro* model of NAFLD
280 (9), and cultured HepG2 cells with either palmitate or oleate. It must be noted that, although HepG2
281 cells are derived from liver hepatocellular carcinoma and they express TRAIL receptors
282 (**Supplementary Figure 2B**), they are normally resistant to TRAIL-induced apoptosis (25). MTT assay
283 showed that palmitate significantly impaired HepG2 cell viability, while oleic acid had no effect on it
284 (**Figure 5A**). In HepG2 cells cultured with oleic acid, TRAIL treatment promoted cell viability (**Figure**
285 **5A**), which is consistent with earlier observations that TRAIL can also activate survival pathways (26).
286 Most importantly, in these cells, TRAIL treatment reduced lipid droplet accumulation by 45% (**Figure**
287 **5B-C**). This effect was confirmed in primary hepatocytes, where TRAIL treatment reduced lipid
288 droplet accumulation by 39% (**Figure 5B-C**). In addition, in HepG2 cells cultured with oleic acid,
289 TRAIL upregulated *PPAR γ* , *PGC-1 α* , and *ATG7* gene expression (**Figure 6A-C**), as well as PGC-1 α
290 protein expression (**Figure 6D**), while it had no effect on specific proinflammatory markers
291 (**Supplementary Figure 2C**).

292

293 **Discussion**

294 The first new finding of this study is that TRAIL significantly ameliorated diet-induced metabolic
295 abnormalities even when it was administered after the development of impaired glucose tolerance. At
296 the end of the study, HFD mice became obese and insulin resistant, displaying impaired glucose
297 tolerance as well as an increase in NEFA and CRP levels. By comparison, the group of HFD+TRAIL
298 mice showed a significant reduction in body weight gain, NEFA and CRP levels, as well as a
299 significant amelioration of insulin resistance and impaired glucose tolerance.

300 Looking at the adipose tissue, TRAIL reduced body weight gain, which was associated with a decrease
301 in fat weight, adipocyte size, and pgWAT macrophage infiltration. These findings are consistent with
302 the earlier observations that TRAIL had significant effects on body weight. As a matter of fact, genetic
303 TRAIL deficiency was found to be associated with increased body weight (8), and we reported that
304 TRAIL treatment significantly reduced the adiposity of HFD-fed mice as assessed by EchoMRI (7).
305 More recently, it has been shown that TRAIL has the ability to inhibit adipogenic differentiation
306 through caspase activation (27). When interpreting our results in light of this finding, it is also by
307 reducing fat mass that TRAIL could have ameliorated insulin resistance, impaired glucose tolerance, as
308 well as circulating NEFA and CRP in HFD mice. Excess fat mass has been associated not only with
309 insulin resistance (20), but also with high levels of NEFA (20) and a low-grade systemic inflammatory
310 state (21). Moreover, experimental studies have shown that insulin sensitivity and systemic
311 inflammation improve following adipose tissue removal (28).

312 In this study, HFD mice developed also hepatic steatosis and inflammation, corresponding to human
313 non-alcoholic fatty liver disease (NAFLD). At present, NAFLD is found to be a frequent comorbid
314 factor in the setting of type 2 diabetes (29, 30). It is estimated that about 70% of obese patients with
315 diabetes have NAFLD and as many as 30-40% have non-alcoholic steatohepatitis (NASH), which is

316 characterized by hepatic steatosis with inflammation and/or necrosis (30). Both NAFLD and NASH are
317 conditions leading to hepatic cirrhosis, end-stage liver disease, and hepatocellular carcinoma (29).
318 Given that NAFLD is reaching epidemic proportions in diabetic patients (29, 30), it is predicted that
319 cirrhosis related to NASH will surpass HCV-related cirrhosis as the most common indication for liver
320 transplantation in the United States (31).

321 Therefore, the second important finding of this study is that TRAIL treatment markedly reduced
322 NAFLD in the HFD-fed mouse, where it decreased liver fat content by 47%. Given that NAFLD
323 generally promotes metabolic abnormalities (29, 30), it is also by reducing liver fat content that TRAIL
324 might have ameliorated insulin resistance and subclinical inflammation in HFD-fed mice. To date, only
325 a few studies have addressed the relationship between TRAIL and NAFLD (9, 32). By comparison, this
326 is the first study describing the direct effect of TRAIL in an experimental model of NAFLD.
327 Nevertheless, our data is consistent with the finding that TRAIL deficiency worsens NAFLD (9).

328 There seem to be several mechanisms underlying TRAIL actions on the liver. First, the reduction in
329 body weight gain, fat mass, and circulating NEFA, which were induced by TRAIL, could have
330 ameliorated NAFLD in the HFD+TRAIL group. It has been argued that excess storage of hepatic
331 triglycerides comes mostly from an excess of circulating NEFA (33), which is usually associated with
332 visceral obesity (34) or adipose tissue insulin resistance/inflammation (34, 35). Second, the significant
333 reduction of insulin, which was observed in the HFD+TRAIL group, could have also contributed to
334 NAFLD amelioration. Hyperinsulinemia usually contributes to liver steatosis. Insulin promotes the
335 synthesis and inhibits the degradation of lipids (36). It stimulates key lipogenic genes in the liver, such
336 as fatty acid synthase (FAS), while reducing CPT1, which is the transporter of NEFA into
337 mitochondria, thereby reducing fatty acid oxidation (36). FAS inhibitors have proven useful to reduce
338 liver triglyceride content (37). In this study, HFD mice displayed an upregulation of *Fas* and a

339 downregulation of *Cpt1a* in the liver. TRAIL significantly reduced HFD-induced *Fas* upregulation,
340 consistent with the reduction of insulin and liver steatosis. Third, this study clearly shows that TRAIL
341 also has direct actions on hepatocytes, where it significantly decreased fat content.

342 In this study, TRAIL significantly reduced lipid droplet accumulation in both HepG2 cells and primary
343 hepatocytes cultured with oleate. Our results are consistent with the observation that TRAIL treatment
344 reduces palmitate-induced lipid uptake by 30% in hepatocytes (9). Interestingly, in this study, TRAIL
345 promoted hepatocyte cell viability and did not have anti-inflammatory effects *in vitro*, suggesting that
346 the *in vivo* reduction of liver inflammation might be secondary to the amelioration of steatosis. In
347 addition, TRAIL showed a direct stimulatory effect on liver *Pparγ* and PGC-1 α expression *in vivo*,
348 which was confirmed *in vitro*, where TRAIL significantly increased *PPARγ* and PGC-1 α expression in
349 HepG2 cells. *PPARγ* is a nuclear receptor expressed in adipose tissue, muscle, and liver. Several
350 studies have shown that *PPARγ* agonists significantly reduce hepatic triglyceride content and NAFLD
351 in patients with diabetes (38, 39). There is a functional interaction between *PPARγ* and PGC-1 α , which
352 might explain the parallel increase of their gene expression induced by TRAIL (40, 41). PGC-1 α ,
353 which is a transcriptional coactivator of nuclear receptors, is currently considered as a key component
354 of regulatory networks that control cellular actions to adapt to higher cellular demands, such as
355 mitochondrial function, gluconeogenesis and glucose transport, glycogenolysis, and fatty acid
356 oxidation (42). Interestingly, PGC-1 α polymorphisms have been associated with obesity and increased
357 risk of diabetes (43, 44). Moreover, both PGC-1 α (45) and PGC-1 α -responsive genes are coordinately
358 downregulated in human diabetes (46). When looking at the liver, it has been shown that PGC-1 α -
359 deficient mice (47) and liver-specific PGC-1 α heterozygous mice (48) develop hepatic steatosis.
360 Moreover, PGC-1 α seems to have a suppressive effect on liver inflammation (49). Therefore, our
361 results suggest that TRAIL effects on the liver and glucose metabolism might involve *PPARγ* and/or
362 PGC-1 α actions.

363 In addition, based on our *in vitro* studies, where TRAIL increased *ATG7*, a possible unifying
364 hypothesis explaining TRAIL effects on liver steatosis and PGC-1 α is that they might be due to
365 TRAIL-induced liver autophagy (50). Autophagy is a mechanism by which TG content, lipid droplet
366 number and size is regulated. In this process, lipids are sequestered in autophagosomes where they are
367 degraded (24). This should stimulate mitochondrial β -oxidation (24). Moreover, autophagy seems to
368 regulate the flux of cholesterol out of the cell to APOA-I (51), which is the major component of HDL
369 and could explain the increase in HDL cholesterol that we found in HFD+TRAIL mice. Further studies
370 are needed to evaluate the mechanisms underlying TRAIL effect on liver steatosis and PGC-1 α , as well
371 as to test in detail the hypothesis that they might include TRAIL actions on liver autophagy.

372 In conclusion, this study shows that TRAIL was effective in reducing HFD-induced metabolic
373 abnormalities even when it was administered after the development of impaired glucose tolerance.
374 Second, this study shows that TRAIL markedly improved NAFLD in HFD-fed mice, and that this was
375 associated with a significant increase in PGC-1 α . Overall, our results shed light on the therapeutic
376 potential of TRAIL against diabetes, NAFLD, and NASH.

377

378 **Perspective**

- 379 • Experimental evidence suggests that a circulating protein called TRAIL protects against type 2
380 diabetes. This study was designed to evaluate whether TRAIL had the potential not only to
381 prevent, but also to treat diet-induced type 2 diabetes.
- 382 • Our *in vivo* results show that TRAIL had the ability to attenuate the metabolic abnormalities
383 induced by a high-fat diet, also when TRAIL was given after disease onset (after 4 weeks of
384 high-fat diet). In particular, TRAIL treatment significantly reduced body weight, impaired
385 glucose tolerance, and liver steatosis, all of which are frequently associated with type 2
386 diabetes.
- 387 • Our *in vitro* results show that TRAIL has direct effects on hepatocytes, where it reduces lipid
388 droplet accumulation. This data sheds light on TRAIL therapeutic potential against impaired
389 glucose tolerance and NAFLD.

390

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395 **Declarations of interest**

396 All Authors have no conflicts of interest.

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401 **Author contribution**

402 S. Bernardi conception and design, acquisition, analysis and interpretation of data, and drafting the
403 article; B.T. acquisition, analysis and interpretation of data and drafting article; V.T. F.B. S. Biffi and
404 A.L. acquisition, analysis and interpretation of data; G.Z. P.S. conception, interpretation of data and
405 article revision for important intellectual content; B.F. conception and design, and article revision for
406 important intellectual content. All the authors have read and approved the submission of this
407 manuscript.

408

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532

533

534 **Tables**535 **Table 1. Liver gene expression analysis**

	CNT	HFD	HFD + TRAIL	HFD vs CNT	HFD + TRAIL vs CNT	HFD vs HFD + TRAIL
<i>De novo lipogenesis</i>						
<i>Fas</i>	1.00 ± 0.10	1.60 ± 0.11	1.20 ± 0.07	<0.01	NS	<0.05
<i>Srebp1a</i>	1.00 ± 0.04	1.17 ± 0.10	1.16 ± 0.11	NS	NS	NS
<i>Srebp1c</i>	1.00 ± 0.20	0.70 ± 0.06	1.17 ± 0.21	NS	NS	NS
<i>Fatty acid oxidation</i>						
<i>Aox</i>	1.00 ± 0.08	0.77 ± 0.06	0.83 ± 0.07	NS	NS	NS
<i>Cpt1a</i>	1.00 ± 0.06	0.51 ± 0.07	0.55 ± 0.07	<0.0001	<0.0001	NS
<i>Ppara</i>	1.00 ± 0.11	0.75 ± 0.14	0.85 ± 0.15	NS	NS	NS
<i>Gluconeogenesis, insulin signaling, insulin sensitivity</i>						
<i>Irs2</i>	1.00 ± 0.09	0.79 ± 0.08	0.74 ± 0.12	NS	NS	NS
<i>Pepck</i>	1.00 ± 0.10	0.30 ± 0.09	0.32 ± 0.06	<0.0001	<0.0001	NS
<i>Pparγ</i>	1.00 ± 0.10	1.82 ± 0.21	2.57 ± 0.20	<0.01	<0.0001	<0.05
<i>Hnf4</i>	1.00 ± 0.11	0.99 ± 0.11	1.07 ± 0.09	NS	NS	NS
<i>Mitochondrial function</i>						
<i>Cit synt</i>	1.00 ± 0.09	1.00 ± 0.11	1.12 ± 0.08	NS	NS	NS
<i>Pgc-1α</i>	1.00 ± 0.05	0.41 ± 0.05	0.65 ± 0.03	<0.0001	<0.0001	<0.01
<i>Pln5</i>	1.00 ± 0.21	0.72 ± 0.07	0.81 ± 0.08	NS	NS	NS
<i>Ucp2</i>	1.00 ± 0.11	0.69 ± 0.07	0.79 ± 0.11	NS	NS	NS
<i>Sirt-1</i>	1.00 ± 0.07	1.06 ± 0.10	1.03 ± 0.06	NS	NS	NS
<i>Oxidative stress and inflammation</i>						
<i>Gp91phox</i>	1.00 ± 0.13	0.83 ± 0.15	0.86 ± 0.16	NS	NS	NS
<i>Il-6</i>	1.00 ± 0.18	1.74 ± 0.14	1.16 ± 0.10	<0.01	NS	<0.05
<i>Mcp1</i>	1.00 ± 0.20	1.95 ± 0.25	1.99 ± 0.38	NS	NS	NS
<i>Tnfα</i>	1.00 ± 0.14	0.82 ± 0.12	1.14 ± 0.23	NS	NS	NS
<i>Autophagy</i>						
<i>Atg7</i>	1.00 ± 0.06	1.33 ± 0.12	1.53 ± 0.11	<0.05	<0.01	NS

536

537 Fatty acid synthase (*Fas*), sterol regulatory element binding protein-1a (*Srebp1a*), and sterol regulatory
538 element binding protein-1c (*Srebp1c*) are transcription factors and enzymes involved in lipogenesis.
539 Acyl-CoA oxidase (*Aox*), carnitine palmitoyl transferase-1a (*Cpt1a*), and peroxisome proliferator-
540 activated receptors (*Ppara*) are transcription factors and enzymes involved in fatty acid oxidation. *Irs2*
541 regulates insulin signaling; *Pepck* regulates gluconeogenesis; peroxisome proliferator-activated

542 receptory (*Ppar γ*) sensitizes to insulin. Hepatocyte nuclear factor 4 (*Hnf4*) is a transcriptional regulator
543 of gluconeogenic genes. Citrate synthase (*Cit synt*), which is the pace-making enzyme of Krebs cycle,
544 and uncoupling protein2 (*Ucp2*) are localized in mitochondria, while PPAR γ coactivator 1 α (*Pgc-1 α*),
545 perilipin 5 (*Pln5*), and sirtuin-1 (*Sirt-1*) regulate mitochondrial functions. *Gp91phox* is a subunit of
546 NADPH oxidase, which is involved in oxidative stress, interleukin-6 (*Il-6*), monocyte chemoattractant
547 protein-1 (*Mcp1*), and *Tnfa* are proinflammatory mediators. Autophagy-related protein 7 (*Atg7*) is a
548 molecule involved in autophagy.

549

550 **Figure legends**

551 **Figure 1. Treatment protocol.** (A) Schematic illustration of the protocol: C57BL/6J mice were fed
552 either a standard diet (SD; 12% fat) or a high-fat diet (HFD; 60% fat) for 12 weeks. Mice on HFD were
553 treated either with saline (NaCl 0.9%) or TRAIL weekly between week 5 and week 12. Mice on SD
554 were treated with saline. (*) Tolerance tests before drug randomization; (**) Tolerance tests at the end
555 of the study (B) Glucose and insulin tolerance tests performed before drug randomization. Upper
556 figures show blood glucose and its area under the curve (AUC) during an IPGTT, middle figures show
557 serum insulin and its AUC during an IPGTT, lower figures show blood glucose and its AUC during an
558 IPITT (C) Representative images of Cy5.5-TRAIL clearance from the peritoneal cavity, as assessed by
559 acquisition of fluorescence emission from the peritoneum after an IP injection of 10 µg of Cy5.5-
560 TRAIL. Cy5.5-TRAIL is TRAIL that was labeled with N-hydroxysuccinimide ester of the cyanine 5.5.
561 Fluorescence emission was collected at 700 nm. For further details, see (14). (D) Circulating human
562 TRAIL after IP injection of 10 µg of TRAIL.

563 **Figure 2. Body weight, adipose tissue, and inflammation.** (A) Body weight throughout the study.
564 The gray area corresponds to the treatment period. (B) Perigonadal white adipose tissue (pgWAT)
565 weight at the end of the study. (C-D) Frequency distribution and adipocyte area. (E) Representative
566 images of pgWAT H&E staining (upper panel; 12.5x, scale bar 50 µm) and F4/80+ macrophages
567 (lower panels; 25x, scale bar 50 µm). (G) NEFA levels at the end of the study. (H) Total cholesterol
568 (TC), HDL cholesterol (HDL-C), and triglycerides (TG) at the end of the study. (I) C-reactive protein
569 levels at the end of the study. Results are presented as mean ± SEM; * p<0.05 vs CNT; # p<0.05 vs
570 HFD.

571 **Figure 3. Glucose metabolism.** (A-F) Glucose and insulin tolerance tests performed at the end of the
572 study. (A) Blood glucose during an IPGTT; (B) Serum insulin during an IPGTT; (C) Blood glucose

573 during an IPITT; (D) Area under the curve (AUC) of the glucose levels during the IPGTT; (E) AUC of
574 the insulin levels during the IPGTT; (F) AUC of the glucose levels during the IPITT; (G) Fasting
575 glucose and insulin at the end of the study. (H) Representative images of pancreatic sections
576 immunostained for insulin (25x, scale bar 50 μ m). Results are presented as mean \pm SEM; * $p < 0.05$ vs
577 CNT; # $p < 0.05$ vs HFD.

578 **Figure 4. Liver changes.** (A) Liver triglyceride content; (B) Percentage of hepatic area positive for fat
579 (stained red) with Oli-Red-O (ORO). (C) Representative images of liver ORO staining (25x, scale bar
580 50 μ m), Picrosirius Red (Sirius Red) staining (12.5X, scale bar 100 μ m), CD68+ macrophages (12.5X,
581 scale bar 100 μ m), and PGC-1 α staining (12.5X, scale bar 100 μ m). (D) Quantification of CD68+ cells
582 (brown nuclei)/area liver tissue. (E) Liver mRNA expression of *Il-6*. *Il-6* is for interleukin-6. mRNA
583 expression is reported as fold induction standardized to the mRNA expression in CNT mice. (F) Liver
584 mRNA expression of *Pgc-1 α* . *Pgc-1 α* is for peroxisome proliferator-activated receptor- γ coactivator-1
585 alpha. mRNA expression is reported as fold induction standardized to the mRNA expression in CNT
586 mice. (G) Quantification of PGC-1 α + staining (brown)/area of liver tissue. Results are presented as
587 mean \pm SEM; * $p < 0.05$ vs CNT; # $p < 0.05$ vs HFD.

588 **Figure 5. Hepatic lipid droplet accumulation *in vitro*.** (A) HepG2 cell viability after exposure to
589 palmitic acid or oleic acid in presence or absence of TRAIL. (B) HepG2 and mPH percentage of cell
590 surface positive for lipid droplets (dark red staining) with Oil-Red-O (ORO). Cells were incubated with
591 oleic acid (250 μ M) in presence or absence of TRAIL (1 ng/mL). HepG2 is for HepG2 cells and mPH
592 is for mouse primary hepatocytes. (C) Representative images of HepG2 cells (upper panel) and mouse
593 primary hepatocytes (lower panel) stained with ORO (20x, scale bar 100 μ m). Results are presented as
594 mean \pm SEM; * $p < 0.05$ vs control; # $p < 0.05$ vs oleic acid without TRAIL.

595 **Figure 6. In vitro studies on HepG2 cells.** (A) HepG2 cell mRNA expression of *PPAR* γ ; (B) *PGC-1* α ;
596 (C) *ATG7*. (D) Representative images of PGC-1 α immunostaining on HepG2 cells. Results are
597 presented as mean \pm SEM; * $p < 0.05$ vs control without TRAIL.

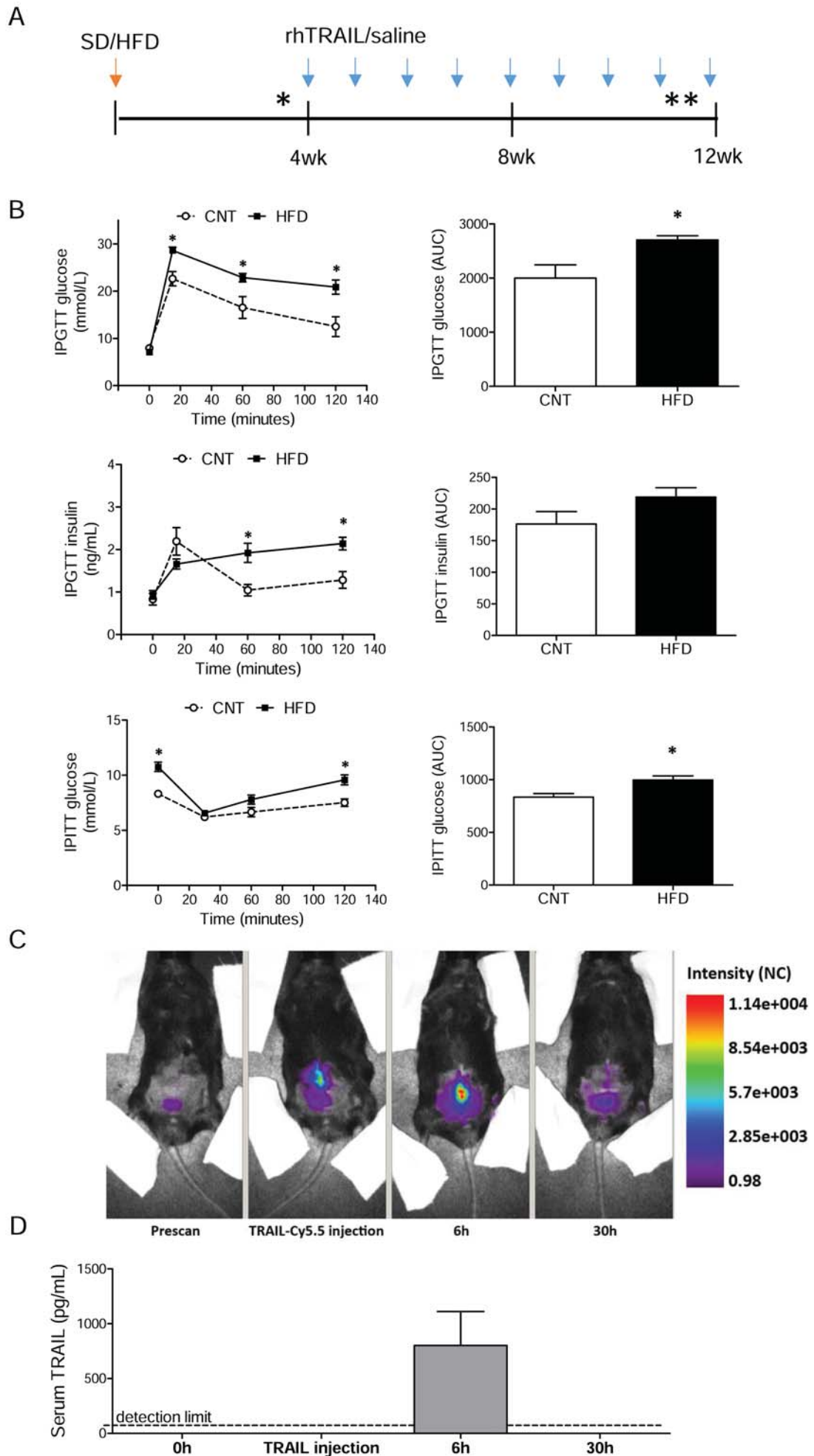


Figure 1

Figure 2

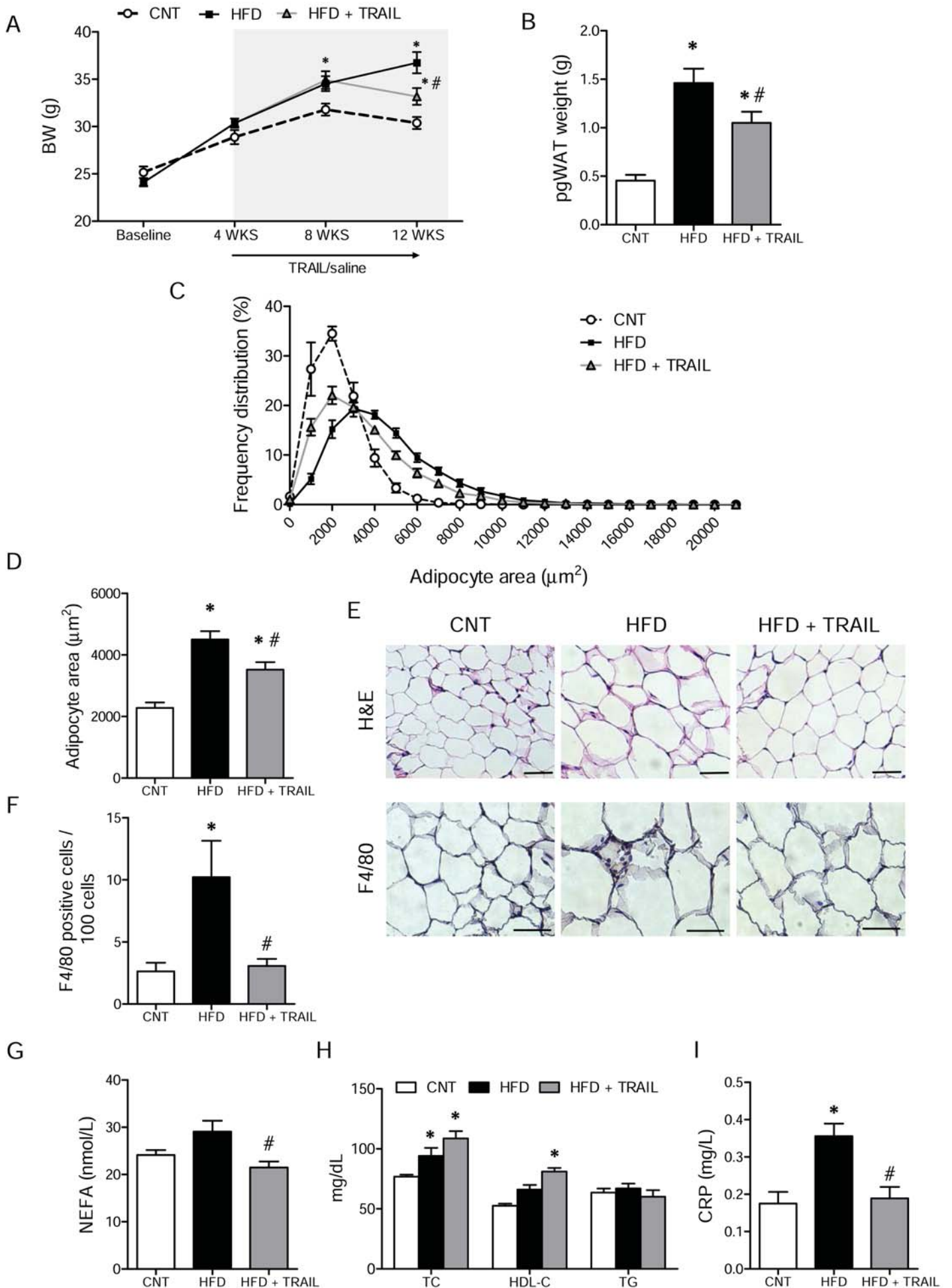


Figure 3

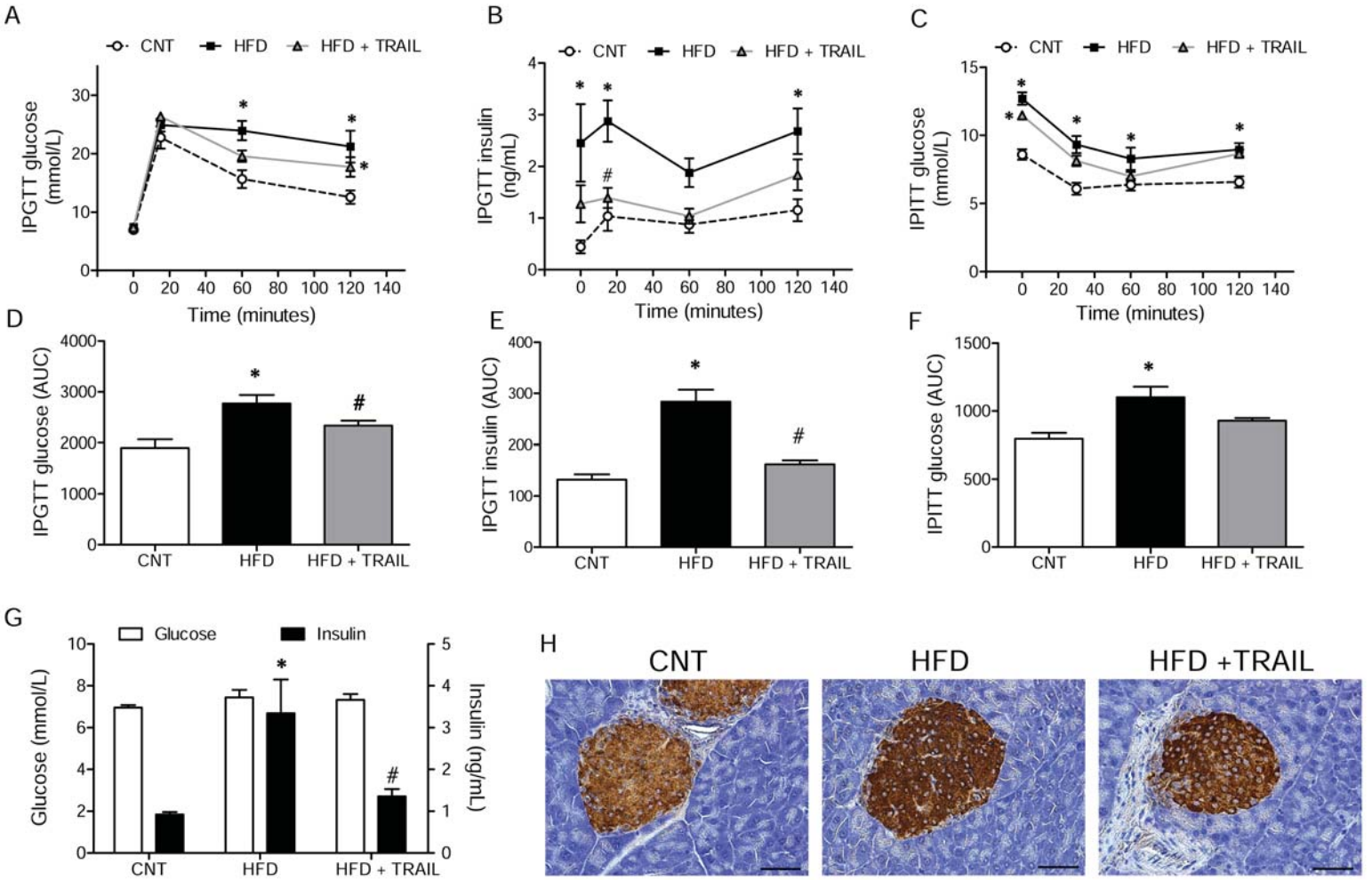


Figure 4

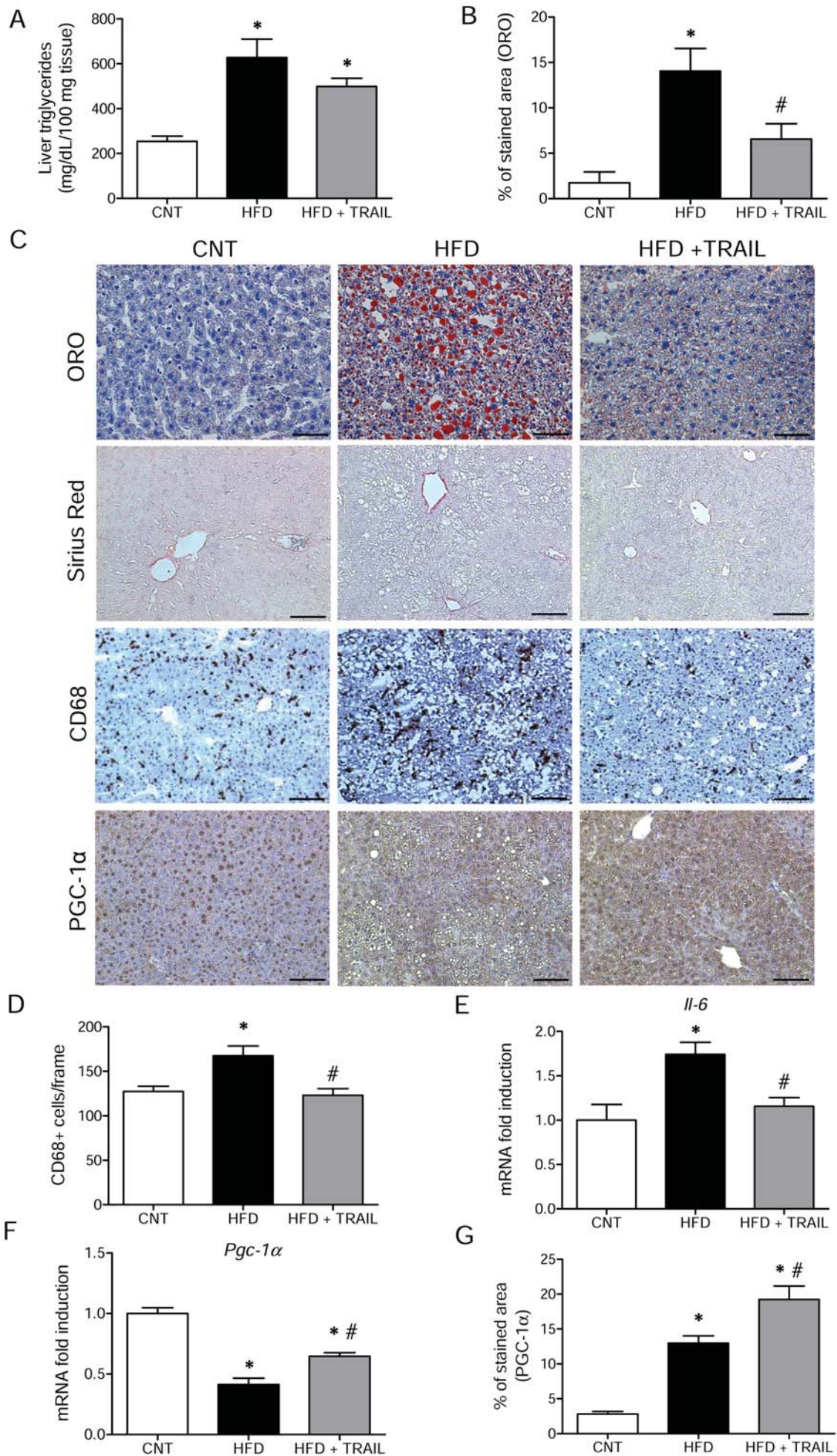


Figure 5

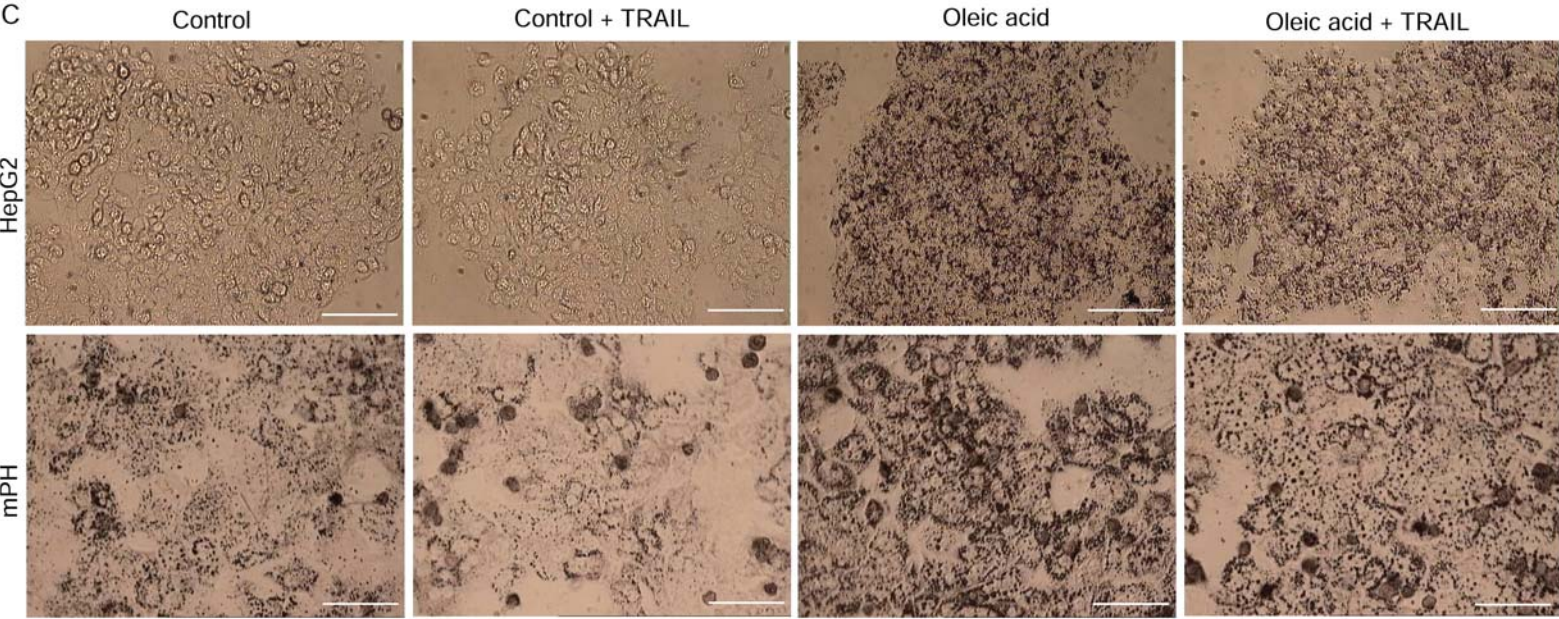
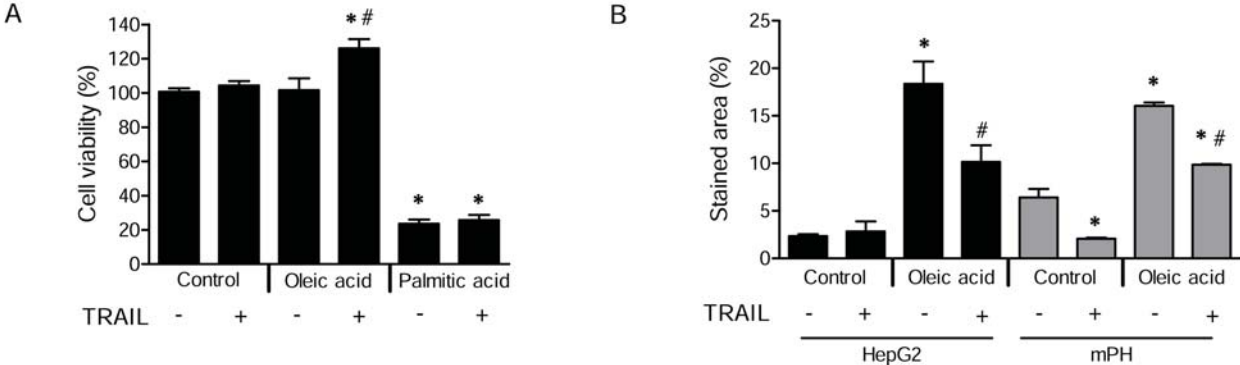
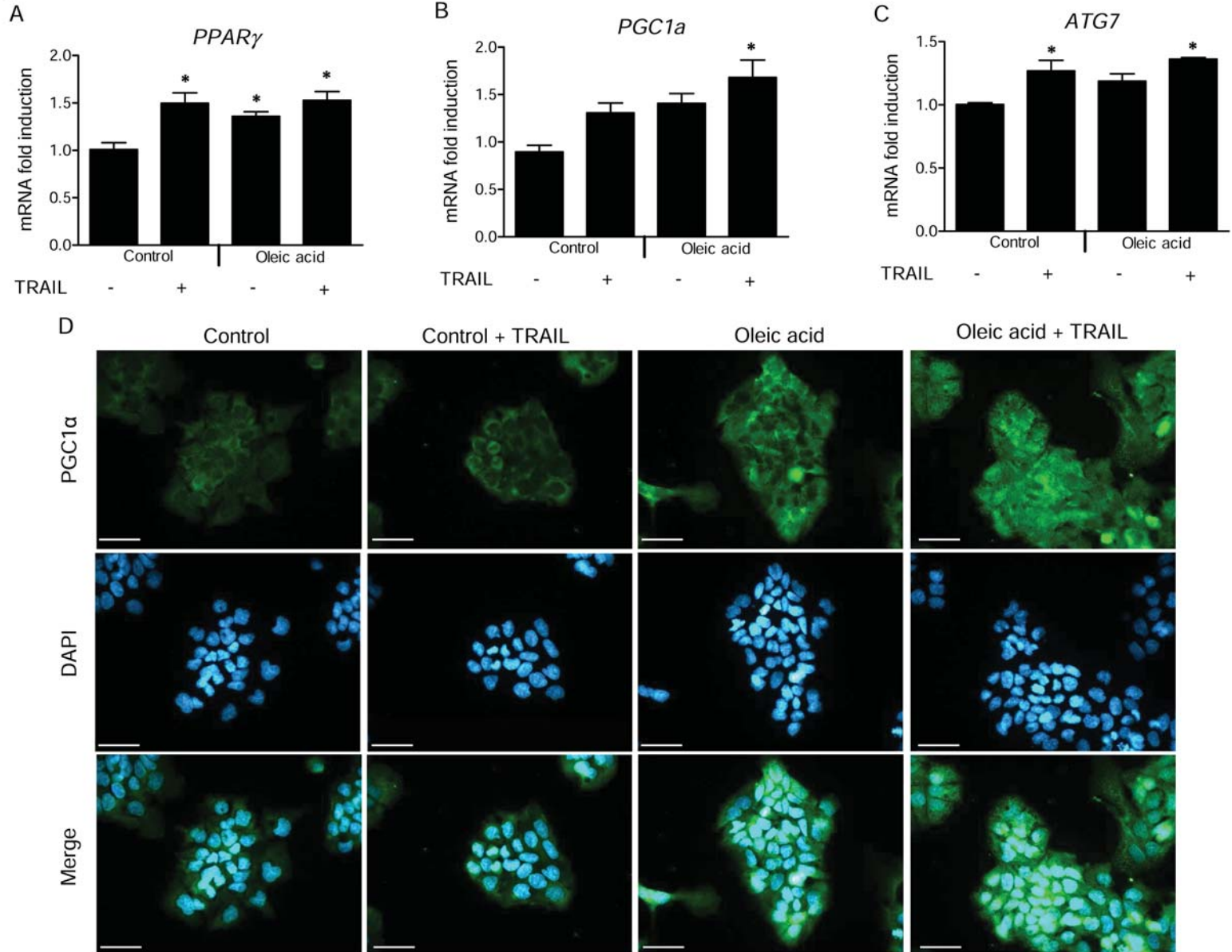


Figure 6



Supplementary Materials and Methods

Recombinant human TRAIL (rhTRAIL) preparation

Recombinant soluble human TRAIL (His6-tagged TRAIL) was produced in bacteria as described by MacFarlane (1997) and purified by affinity chromatography on Ni²⁺ affinity resin (Qiagen, Hilden, Germany). Determination of total protein concentration in the fractions collected after dialysis was performed using a bicinchoninic protein assay with bovine serum albumin as the standard, accordingly to the manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, USA). TRAIL concentration was then quantified by ELISA (R&D Systems, Minneapolis, Minn., USA; sensitivity: 2.9 pg/ml; intra- and interassay CV were 3.9 and 6%, respectively) in duplicate samples. Selected samples were run in each ELISA plate as internal controls to confirm the reproducibility of the determinations over time. The functional activity of each TRAIL preparation was tested on the TRAIL-sensitive HL60 cell line by evaluating cell viability and apoptosis after treatment of the cells with serial dilutions of each preparation. Finally, purified TRAIL batches were tested for the absence of endotoxin by using the ToxinSensor chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ) and stored at -80°C in single use aliquots.

Labeling of rhTRAIL

Purified rhTRAIL was labeled with N-hydroxysuccinimide ester of the cyanine 5.5 (Amersham Biosciences; FluorolinkCy5.5 Monofunctional Dye 5-pack) to form TRAIL-Cy5.5. A freshly prepared solution of dye (0.05 mg/mL) in 0.1M sodium carbonate buffer pH 8 was added to rhTRAIL (1 mg/mL) in phosphate buffer (vol/vol 1:1). The reagents were incubated by gently shaking for 1 hour at room temperature. Excess unconjugated dye was removed by overnight dialysis against phosphate buffer pH 7.4.

Triglyceride measurement with the Beckman Coulter AU5800 analyzer

The procedure for the determination of triglyceride concentration with the Beckman Coulter AU5800 analyzer is based on a series of coupled enzymatic reactions. The triglyceride in the sample are hydrolyzed by a combination of microbial lipases to give glycerol and fatty acids. The glycerol is phosphorylated by ATP in the presence of glycerol kinase to produce glycerol-3-phosphate. The glycerol-3-phosphate is oxidized by molecular oxygen in the presence of glycerol phosphate oxidase to produce hydrogen peroxide (H_2O_2) and dihydroxyacetone phosphate. The formed H_2O_2 reacts with 4-aminophenazone and N,N-bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt (MADB) in the presence of peroxidase (POD) to produce a chromophore, which is red at 660/880 nm. The increase in absorbance at 660/800 nm is proportional to the triglyceride content of the sample.

Supplementary Table 1. Sequences of probes/primer pairs

Gene	Primer pair
Mouse	
<i>Rps9</i>	(F) 5'-GACCAGGAGCTAAAGTTGATTGGA-3' (R) 5'-TCTTGGCCAGGGTAAACTTGA-3'
<i>Fas</i>	(F) 5'-TCGTGATGAACGTGTACCGG-3' (R) 5'-GGGTGAGGACGTTTACAAAG-3'
<i>Srebp1a</i>	(F) 5'-ATGGACGAGCTGGCCTTCG-3' (R) 5'-TGTTGATGAGCTGGAGCATGTCTTC-3'
<i>Srebp1c</i>	(F) 5'-ATGGATTGCACATTTGAAGACATGCT-3' (R) 5'-CCTGTGTCCCCTGTCTCAC-3'
<i>Aox</i>	(F) 5'-TCACGTTTACCCCGGC-3' (R) 5'-CAAGTACGACACCATACCAC-3'
<i>Cpt1a</i>	(F) 5'-CCAAGTATCTGGCAGTCGA-3' (R) 5'-CGCCACAGGACACATAGT-3'
<i>Ppara</i>	(F) 5'-TCAAGGTGTGGCCCAAGGTTA-3' (R) 5'-CGAATGTTCTCAGAAGCCAGCTC-3'
<i>Irs2</i>	(F) 5'-GACTTCCTGTCCCATCACTTG-3' (R) 5'-TTTCAACATGGCGGCGA-3'
<i>Pepck</i>	(F) 5'-CCATCCCAACTCGAGATTCTG-3' (R) 5'-CTGAGGGCTTCATAGACAAGG-3'
<i>Pparγ</i>	(F) 5'-TGTCGGTTTCAGAAGTGCCTTG-3' (R) 5'-TTCAGCTGGTCGATATCACTGGAG-3'
<i>Hnf4</i>	(F) 5'-CAAGAGGTCCATGGTGTTTAAGG-3' (R) 5'-CGGCTCATCTCCGCTAGCT-3'
<i>Cit Synt</i>	(F) 5'-CAAGCAGCAACATGGGAAGA-3' (R) 5'-GTCAGGATCAAGAACCGAAGTCT-3'
<i>Pgc-1α</i>	(F) 5'-TGATGTGAATGACTTGGATACAGACA-3' (R) 5'-GCTCATTGTTGTAAGTGGATATG-3'
<i>Ucp2</i>	(F) 5'-GCCTCTGGAAAGGGAGTTCTC-3' (R) 5'-ACCAGCTCAGCACAGTTGACA-3'
<i>gp91phox</i>	(F) 5'-TTGGGTCAGCACTGGCTCTG-3' (R) 5'-TGCGGTGTGCAGTGCTATC-3'
<i>Il-6</i>	(F) 5'-ACCAGAGGAAATTTCAATAGGC-3' (R) 5'-TGATGCACTTGCAGAAAACA-3'
<i>Tnfa</i>	(F) 5'-AAGCCTGTAGCCACGTCGTA-3' (R) 5'-GGCACCCTAGTTGGTTGTCTTTG-3'
<i>Atg7</i>	(F) 5'-TGCCTATGATGATCTGTGTC-3' (R) 5'-CACCAACTGTTATCTTTGTCC-3'
<i>Pln5</i>	(F) 5'-TCTCGCCTATGAACACTCTTTG-3' (R) 5'-GGGATGGAAAGTAGGGCTAG-3'
<i>Sirt-1</i>	(F) 5'-TGTGAAGTTACTGCAGGAGTG-3' (R) 5'-CAAGGCGAGCATAGATACCG-3'
Human	

<i>RPL27</i>	(R) 5'-TGCCTGGCTGGACGCTACT-3' (F) 5'-CTGAGGTGCCATCATCAATGTT-3'
<i>DR4</i>	(F) 5'-CACAAGACCTTCAAGTTTGTGC-3' (R) 5'-TGGACACAACCTCCCAAAG-3'
<i>DR5</i>	(F) 5'-ATCGTGAGTATCTTGACGCC-3' (R) 5'-TGAGACCTTTCAGCTTCTGC-3'
<i>DcR1</i>	(F) 5'-GATTACACCAACGCTTCCAAC-3' (R) 5'-TGCCTTCTTTACACTGACACAC-3'
<i>DcR2</i>	(F) 5'-GTGGTTGTGGTTGGCTTTTC-3' (R) 5'-CAGGAACCTCGTAAGGACATG-3'
<i>PPARγ</i>	(F) 5'-AAGGCGAGGGCGATCTTG-3' (R) 5'-CCCATCATTAAAGGAATTCATGTCA-3'
<i>PGC-1α</i>	(F) 5'-AAACAGCAGCAGAGACAAATGC-3' (R) 5'-TTGGTTTGGCTTGTAAAGTGTG-3'
<i>ATG7</i>	(F) 5'-TCGAAAGCCATGATGTCGTCTT-3' (R) 5'-CCAAAGCAGCATTGATGACCA-3'
<i>CXCL8</i>	(F) 5'-TTCCTGATTTCTGCAGCTCT-3' (R) 5'-TGTCTTTATGCACTGACATC-3'
<i>TNFα</i>	(F) 5'-CAGGGACCTCTCTAATCA-3' (R) 5'-GGCTACAGGCTTGCACTCG-3'
Gene	Probe/Primer pair
Mouse	
<i>Mcp1</i>	(P) FAM-5'-TCCCTGTCATGCTTCTGGGCCTGT-3'-TAMRA (F) 5'-CTTCCTCCACCACCATGCA-3' (R) 5'-CCAGCCGGCAACTGTGA-3'

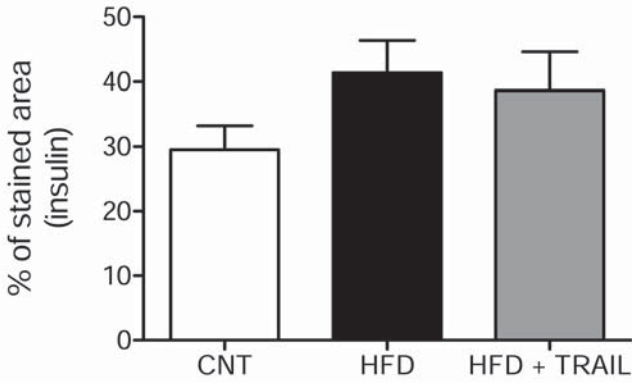
Supplementary Figure Legends

Supplementary Figure 1. Pancreatic beta cell density and mass. (A) Pancreatic beta cell density as assessed by percentage of islet area positive for insulin (brown staining); (B) Pancreatic beta cell mass was estimated by multiplying the mean density of staining for insulin in the islet section by the mean islet area per area of pancreas. This was adjusted for the pancreatic wet weight for individual animals and expressed as fold changes relative to CNT mice.

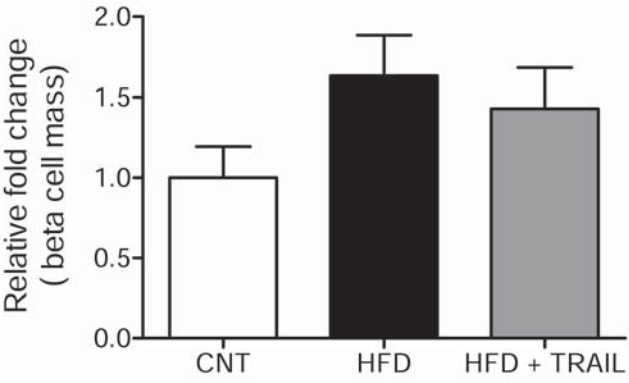
Supplementary Figure 2. Gene expression in *in vivo* and *in vitro* studies. (A) Liver, white adipose tissue (WAT), and skeletal muscle mRNA expression of *Ppar γ* , *Pgc-1 α* , and *Atg7*. PPAR γ is for peroxisome proliferator-activated receptor- γ ; PGC-1 α is for peroxisome proliferator-activated receptor- γ coactivator-1 alpha; ATG7 is for autophagy-related protein 7. mRNA expression is reported as fold induction standardized to the mRNA expression in CNT mice. Results are presented as mean \pm SEM; * $p < 0.05$ vs CNT; # $p < 0.05$ vs HFD. (B-C) HepG2 cell mRNA expression of TRAIL receptors (*DR4*, *DR5*, *DcR1*, *DcR2*) and proinflammatory molecules (*CXCL8* and *TNF α*). DR is for death receptor; DcR is for decoy receptor; CXCL8 is for chemokine (C-X-C motif) ligand 8. Cells were treated with oleic acid (250 μ M) in presence or absence of rhTRAIL (1 ng/mL) for 6 hours. For primer pairs see Supplementary Table 1.

Supplementary Figure 1

A



B



Supplementary Figure 2

