

1 **Ketogenic diet promotes tumor ferroptosis but induces 2 relative corticosterone deficiency that accelerates cachexia**

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31 HIGHLIGHTS

- 32 • Ketogenic diet delays tumor growth but accelerates cancer cachexia and shortens survival
33 • In the tumor, accumulation of lipid peroxidation products results in saturation of the GSH
34 detoxifying pathway and ferroptotic death of cancer cells
35 • In the host organism, systemic redox state imbalance causes NADPH depletion, GDF-15
36 elevations, and relative corticosterone deficiency
37 • Dexamethasone coadministration with ketogenic diet delays onset of cancer cachexia by
38 improving food intake, glucose homeostasis and utilization of nutritional substrates

39

40 SUMMARY

41 The dependency of cancer cells on glucose can be targeted with high-fat low-
42 carbohydrate ketogenic diet (KD). However, hepatic ketogenesis is suppressed in IL-6 producing
43 cancers, which prevents the utilization of this nutrient source as energy for the organism.

44 In two IL-6 associated murine models of cancer cachexia we describe delayed tumor
45 growth but accelerated onset of cancer cachexia and shortened survival when mice are fed KD.
46 Mechanistically, we find this uncoupling is a consequence of the biochemical interaction of two
47 simultaneously occurring NADPH-dependent pathways. Within the tumor, increased production
48 of lipid peroxidation products (LPPs) and, consequently, saturation of the glutathione (GSH)
49 system leads to ferroptotic death of cancer cells. Systemically, redox imbalance and NADPH
50 depletion impairs the biosynthesis of corticosterone, the main regulator of metabolic stress, in
51 the adrenal glands. Administration of dexamethasone, a potent glucocorticoid, improves food
52 intake, normalizes glucose homeostasis and utilization of nutritional substrates, delays onset of
53 cancer cachexia and extends survival of tumor-bearing mice fed KD, while preserving reduced
54 tumor growth.

55 Our study highlights that the outcome of systemic interventions cannot necessarily be
56 extrapolated from the effect on the tumor alone, but that they have to be investigated for anti-
57 cancer and host effects. These findings may be relevant to clinical research efforts that
58 investigate nutritional interventions such as KD in patients with cancer.

59

60 INTRODUCTION

61 Cancer, at a cellular and organismal level, is at least in part a metabolic disease. Cancer
62 cells themselves have altered metabolism to accommodate nutrient demand and maintain
63 growth and proliferation. For example, they frequently rely on increased glucose consumption
64 to supply anabolic metabolism (Warburg 1925). At a systemic level, cancer can also alter the
65 metabolism of the host by inducing profound changes in nutrient intake and handling that
66 culminate in cachexia. Cancer cachexia is a severe wasting syndrome characterized by reduced
67 food intake and terminal weight loss that affects up to 80% of all patients with cancer (von
68 Haehling and Anker 2014), and causes significant morbidity and mortality (Farkas et al. 2013).
69 The involuntary weight loss suffered by patients with cancer cachexia cannot be reversed by
70 nutritional supplementation (Fearon et al. 2011), and this persistent metabolic stress condition
71 increases glucocorticoids levels in humans and mouse models of cancer cachexia.

72 The dependency of cancer cells on glucose has been targeted by utilization of ketogenic
73 diets (KD) containing high levels of fats and low levels of carbohydrates. KDs are explored as
74 therapeutic intervention in end-stages of cancer that are associated with cachexia (Jansen and
75 Walach 2016). Several studies report an anti-inflammatory and a delayed tumor growth effect
76 of KD in pre-clinical models (Nakamura et al. 2018; Otto et al. 2008; Seyfried et al. 2003) and in
77 humans (Schwartz et al. 2015). However, hepatic ketogenesis is suppressed in murine models
78 of cancer progression that are associated with interleukin-6 (IL-6) elevation and ultimately
79 cause cancer cachexia (Flint et al. 2016; Goncalves et al. 2018). This results in an inability of the
80 organism to convert KD to survival-sustaining energy supply molecules. Together this raises the
81 question whether the inability of the cancer cell or the organism to utilize the macronutrients
82 supplied by KD is dominant with regard to outcome.

83 Lipid peroxidation is a non-enzymatic route of fatty acid metabolism that is oxygen
84 radical-dependent (Massey and Nicolaou 2011; Yin, Xu, and Porter 2011). Metabolism of fats
85 through non-enzymatic lipid peroxidation is a recognized source of highly reactive molecules
86 named lipid peroxidation products (LPPs), such as 4-hydroxynonenal (4-HNE) and
87 malondialdehyde (MDA), that cause cross-linkage on DNA and proteins through the formation
88 of etheno-adducts (Goldstein 1975; Nair et al. 2019). Under physiological conditions, lipid

89 peroxidation rates are low and non-toxic since LPPs are quickly removed from cells by
90 constitutive antioxidants defense systems such as the NADPH-dependent glutathione (GSH)
91 system (Little and O'Brien 1968; Ursini et al. 1982). When LPP detoxification fails, the
92 accumulation of lipid peroxides results in a type of programmed cell death dependent on iron
93 that is termed ferroptosis (Yang et al. 2014).

94 Cortisol is the major human glucocorticoid, equivalent to corticosterone in rodents.
95 Cortisol release is part of the physiological response to starvation in cancer cachexia that drives
96 adaptive pathways and regulates nutrient storage and processing, glucose levels, protein
97 breakdown (Hoberman 1950; Wing and Goldberg 1993), and lipolysis (Divertie, Jensen, and
98 Miles 1991). Biosynthesis of glucocorticoids occurs in the cortex of the adrenal gland through
99 repeated NADPH-dependent enzymatic reduction of cholesterol. This process is under the
100 control of the hypothalamic-pituitary-adrenal (HPA) axis. The inability to mount an adequate
101 stress response due to irreversible damage to the adrenal cortex (e.g., auto-immunity)
102 (Bratland et al. 2009) or pharmacotherapy-induced suppression of the HPA axis (Henzen et al.
103 2000) presents a life-threatening condition.

104 Therefore, glucocorticoid synthesis and the pathway of LPP detoxification share the
105 requirement for NADPH as cofactor, yet their biochemical interdependency has not been
106 explored. This interaction becomes relevant when both pathways simultaneously occur in
107 metabolically stressed organisms (e.g., cachexia) fed a diet with high fat content (e.g., ketogenic
108 diet).

109 In this study, we set out to determine the differential effect of KD on tumors and the
110 host organism using two murine models of cancer cachexia. We find that although KD slows
111 tumor growth, it shortens survival by accelerating the onset of cachexia. Mechanistically,
112 increased production of LPPs in KD-fed tumor-bearing mice leads to a systemic redox state
113 imbalance. Within tumors, this results in saturation of the GSH detoxifying pathway and
114 consequent ferroptotic death of cancer cells. Moreover, we discover that NADPH depletion
115 impairs corticosterone biosynthesis in the adrenal cortex, inducing a relative
116 hypocorticosteronemia and metabolic maladaptation in mice fed KD. Treatment with the
117 synthetic corticosteroid dexamethasone delays the onset of cancer cachexia and extends

118 survival of tumor-bearing mice fed KD by improving food intake, metabolic homeostasis and
119 utilization of nutritional substrates while preserving reduced tumor growth.

120 The uncoupling of tumor growth from overall survival illustrates why clinical trials
121 should monitor the host response to nutritional interventions such as KD closely.

122

123 RESULTS

124 **Ketogenic diet delays tumor growth but shortens overall survival in two mouse models of**
125 **cancer cachexia**

126 To investigate the effect of ketogenic diet on established IL-6-secreting cachexia-
127 inducing cancers and the tumor-bearing host, BALB/c mice bearing subcutaneous C26
128 colorectal tumors for 14 days, and KPC mice ($\text{Kras}^{\text{G12D}+/+}$; $\text{Trp53}^{\text{R172H}+/+}$; Pdx-1-Cre), a genetically
129 engineered mouse model (GEMM) of pancreatic cancer, with >3-5mm size tumors were
130 challenged with a low-carbohydrate, moderate-protein, high-fat diet (KD) or maintained on
131 normal diet feeding (NF) (Figure S1A and Table S1). Tumor growth was significantly decelerated
132 in mice fed KD in both models, indicating a diet-mediated anti-tumor effect (Figures 1A and 1B).
133 However, KD prompted an earlier onset of cancer cachexia (>15% bodyweight loss) and
134 systemic wasting, thus shortening overall survival (OS) in both C26 and KPC mice fed KD
135 compared to their counterparts fed NF (Median OS: 10 days C26/KD, 14 days C26/NF, 6.5 days
136 KPC/KD, 17 days KPC/NF) (Figures 1C and 1D, Figures S1B and S1C). At endpoint, tumor-bearing
137 mice exhibited loss of subcutaneous and gonadal fat tissue, depletion of quadriceps muscle
138 mass and splenomegaly, recognized signs of cachexia (Figures S1D and S1E).

139 Longitudinal monitoring of blood glucose levels in mice bearing established tumors
140 showed an acute decrease in glucose of littermate controls (LM) and PC controls after
141 introduction of KD that completely recovered after 24h to similar levels of controls fed NF. In
142 contrast, C26 and KPC tumor-bearing mice on KD did not adapt to the new nutritional source
143 and their glucose levels kept declining over time, reaching the lowest levels at cachectic
144 endpoint. Tumor-bearing mice on NF had lower glucose levels than their non-tumor bearing
145 counterparts but higher than tumor-bearing mice on KD, and they were able to maintain stable
146 glucose measurements until onset of cachexia when the levels dropped abruptly (Figures 1E

147 and 1F). Circulating ketones were significantly increased in all KD fed compared to NF fed
148 groups, but tumor-bearing mice had lower levels compared to their non-tumor bearing control
149 counterparts on the same diet (Figures 1G and 1H). Littermate mice on KD had a 2-fold
150 upregulation of hepatic mRNA levels of PPAR α target genes that regulate ketogenesis, *Acadm*
151 and *Hmgsc2*, compared to littermates on NF. The expression was also 2-fold higher than in C26
152 tumor-bearers on KD, which were unable to upregulate the transcriptional targets responsible
153 for ketogenesis despite the increased dietary substrate. C26 tumor-bearing mice on NF
154 exhibited suppressed transcriptional regulation of ketogenesis compared to NF fed littermates,
155 as previously described (Flint et al. 2016) (Figures S1F and S1G). Food intake was decreased in a
156 similar manner in both tumor-bearing groups as cachexia developed (Figures S1H and S1I).
157 Taken together, these data demonstrate not only that KD does not overcome the tumor-
158 mediated metabolic reprogramming of the liver that suppresses ketogenesis, but it also impairs
159 metabolic responses that maintain glucose homeostasis in the tumor-bearing host.
160

161 **Ketogenic diet induces formation of etheno-adducts and ferroptotic cell death of cancer cells
162 that can be prevented by NAC**

163 Detoxification of reactive LPPs by the constitutive antioxidant glutathione (GSH) is a
164 NADPH-demanding process (Figure S2A)(Pannala et al. 2013).

165 To quantify the formation of lipid peroxidation products in the context of the lipid-
166 enriched KD (Table S1) and investigate the redox state of both the tumor and the host, we
167 performed an in-depth metabolomics analysis on liver and tumor tissue of tumor-bearing C26
168 mice and control mice fed KD or NF. 4-HNE, the major lipid peroxide resulting from oxidation of
169 fatty acids, accumulated in the liver of tumor-bearing C26 mice fed KD compared to NF fed
170 tumor-bearing mice. Littermates without tumors on KD had unchanged levels of 4-HNE in the
171 liver compared to NF fed littermates, suggesting its production was efficiently detoxified (Figure
172 2A). GSH to GSSG ratio in the liver was decreased in C26 tumor-bearing mice on KD compared
173 to those on NF, suggesting an ongoing utilization of the reductive power of glutathione
174 molecules with the purpose of detoxifying LPPs (Figure 2B). Tumor metabolomics of C26 mice
175 fed KD or NF were separated by PCA (Figure S2B). GSH to GSSG ratio in tumors from C26 mice

176 on KD was lower indicating a diminished reductive potential, in keeping with GSH consumption
177 for the detoxification of LPPs (Figure 2C). The rate-limiting precursor metabolite for GSH
178 biosynthesis, cysteine, was also decreased in tumors from C26 mice fed KD (Figure 2D),
179 whereas ophthalmate, a biomarker for oxidative stress and GSH depletion (Dello et al. 2013),
180 significantly accumulated in KD tumors (Figure S2C). Other indicators of redox perturbation,
181 such as collapse of the antioxidant carnosine (Scuto et al. 2020) (Figure S2D) and evidence of
182 hypotaurine to taurine oxidation (Figures S2E and S2F) (Fontana et al. 2005) were present in
183 tumors of C26 KD-fed mice. These data are compatible with an increased formation of toxic and
184 highly mutagenic lipid peroxides that saturates the GSH pathway in tumors from C26 mice on
185 KD compared to those fed NF. Adduct formation by the lipid peroxide 4-HNE was significantly
186 elevated in the tumors of C26 and KPC tumor-bearing mice fed KD compared to those fed NF
187 (Figure 2E and 2F). 4-HNE adducts in KD-fed C26 mice were prevented with administration of N-
188 acetyl cysteine (NAC), an antioxidant that boosts the GSH pathway by increasing GSH
189 biosynthesis and therefore clearance of LPPs (Figures 2E, S2A and S2G).

190 Accumulation of lipid peroxides results in a type of programmed cell death dependent
191 on iron named ferroptosis (Dixon et al. 2012) that has previously been described in cysteine-
192 depleted tumors (Badgley et al. 2020). To explore the possibility that ferroptotic cell death in
193 tumors from KD-fed mice is partly responsible for reduced tumor burden and slower tumor
194 growth trajectories, we first performed Oil-Red-O staining of lipids in tumors from C26 mice.
195 C26 mice on KD had significant accumulation of lipid droplets in the tumor tissue, whereas
196 tumors from C26 mice on NF had no evidence of lipid storage (Figure S2H). We next quantified
197 the levels of ferrous (Fe^{2+} /Iron II) and ferric (Fe^{3+} / Iron III) iron in tumor samples. Ferrous iron is
198 an established indirect marker of ferroptosis and it accumulated in tumor tissues from C26 and
199 KPC mice fed KD, suggesting an ongoing ferroptotic cell death mechanism in these tumors
200 compared to tumors from mice fed NF (Figure 2G and 2H). A dead tumor core was observed in
201 tumors from mice fed KD by hematoxylin and eosin (H&E) staining (Figure 2I), supporting an
202 ongoing cell death process in these tumors. Moreover, tumors of mice on KD treated with NAC
203 were notably bigger than those in untreated mice fed KD (Figure 2J), and ferrous iron levels
204 were depleted upon NAC treatment (Figure 2G), indicating that NAC administration is sufficient

205 to prevent ferroptosis and cell death in these tumors. We performed a pharmacological
206 experiment to support this hypothesis, with the aim to recapitulate the effect induced by KD.
207 Indeed, administration of RSL3, a specific inhibitor of glutathione peroxidase 4 (GPx4) that
208 blocks the detoxification of LPPs by the GSH pathway and thereby induces cellular ferroptosis,
209 to tumor-bearing mice fed NF led to reduced tumor growth (Figure 2K).

210 We next set out to validate these findings in the KPC model. RNAseq data from tumors
211 of cachectic KPC mice demonstrated significant overexpression of E2F and Myc targets, which
212 are major regulators of cellular metabolism in response to stress (Dong et al. 2020), in tumors
213 of KPC mice fed KD compared to KPC fed NF. The E2F axis has previously been associated to a
214 role as promoter of oxidative stress and ferroptosis in neurons, and its silencing leads to
215 prevention of this iron-dependent cell death (Mishima 2021). Myc signalling has also been
216 linked to mediation of, and sensitization to, ferroptotic cell death (Alborzinia et al. 2022; Lu et
217 al. 2021). Gene expression marking activity of the G2/M checkpoint were significantly
218 upregulated in tumors of tumor-bearing KPC mice fed KD, and since the activation of this
219 checkpoint prevents cells from entering mitosis when the DNA is damaged and allows for its
220 repair, this suggests that LPP formation and reactivity in the cancer cells causes DNA
221 crosslinking, adduct formation and consequently cell cycle arrest. The activity of the
222 cytochrome P450 reductase (POR), which has a major role in the metabolism of drugs and
223 xenobiotics, requires NADPH (Esteves, Rueff, and Kranendonk 2021). Downregulation of
224 pathways related to xenobiotic metabolism in the tumors of mice fed ketogenic diet can be
225 explained by the ongoing systemic NADPH depletion in these mice (Figure 2L).

226 Apoptosis was disregarded as an additional mechanism contributing to reduced tumor
227 growth in mice fed KD because apoptotic markers such as Caspase-3 were expressed similarly in
228 Western Blot (WB) of C26 tumors from both dietary groups. BAX staining suggested even lower
229 levels of apoptotic cell death in tumors from C26 KD fed mice compared to those from C26 fed
230 NF (Figure S2I). Cell proliferation, measured by Ki67 staining, was also not affected by the
231 dietary challenge itself (Figure S2J).

232 All of these findings demonstrate that elevated oxidative stress leading to cell cycle
233 arrest and ferroptotic cell death caused by build-up of LPPs are mechanisms contributing to
234 smaller tumor burden in mice fed KD.

235 Since ferroptosis is an immunogenic process (Efimova et al. 2020), we next studied the
236 immune infiltration in tumors from KD- and NF-fed mice. We found a positive trend for
237 enrichment of all immune cell types examined in KD tumors, reaching statistical significance for
238 neutrophils (Figures S2K, S2L, S2M and S2N). This observation may be explained by active
239 recruitment of neutrophils to areas undergoing ferroptotic cell death.

240

241 **Ketogenic diet impairs glucocorticoid synthesis in tumor-bearing mice**

242 Corticosterone is the main glucocorticoid involved in metabolic adaptation under stress
243 conditions in mice that acts as a regulator of metabolic rates and availability of fuel substrates.
244 Metabolic stressors such as caloric restriction associated with cachexia induce high levels of
245 corticosterone (Flint et al. 2016). Similar to the detoxification of LPPs, the corticosteroid
246 synthesis pathway in the cortex of the adrenal gland requires a constant supply of NADPH
247 cofactor molecules (Figure S3A). To gain a deeper understanding of the metabolic stress
248 response in KD and NF fed mice, we quantified circulating levels of corticosterone and
249 cholesterol, the substrate for corticosterone biosynthesis in the adrenals. Control littermates on
250 either diet had baseline normal levels of corticosterone in circulation. At the time of cachexia,
251 tumor-bearing C26 mice fed NF displayed a sharp increase in corticosterone concentration, but
252 levels in tumor-bearing C26 mice fed KD were not elevated in comparison. (Figures 3A and S3B).
253 Cholesterol availability was similar in C26 and KPC mice fed with KD and NF diets (Figures 3B
254 and 3C) but pregnenolone levels significantly accumulated in the plasma of C26 mice fed KD
255 compared to those fed NF (Figure 3D), pointing towards a defect in the synthetic cascade of
256 corticosterone (Figure S3A) rather than an absence of substrate.

257 We next compared the transcriptome of the adrenal glands in tumor-bearing KPC mice
258 fed KD or NF, and using Gene Set Enrichment Analysis (GSEA) we found that the steroid
259 biosynthesis and cholesterol homeostasis pathways were significantly downregulated in KPC
260 mice fed KD compared to those fed NF (Figure S3C). These data demonstrate an impaired

261 corticosterone production and inefficient stress response in the adrenal glands of tumor-
262 bearing mice fed KD compared to those fed NF. One of the major actions of aldosterone, a
263 mineralocorticoid hormone derived from downstream processing of corticosterone in the
264 adrenal glands, is sodium retention and potassium loss. Quantification of circulating sodium in
265 C26 mice and controls revealed a relative hyponatremia in C26 tumor-bearing mice fed KD
266 compared to C26 fed NF and littermate controls (Figures 3E), further supporting impaired
267 hormone biosynthesis in the adrenal glands of these mice.

268 The adrenal glands are part of the HPA axis, which regulates a cascade of endocrine
269 pathways including the production of corticosterone (cortisol in humans). The
270 adrenocorticotropic hormone (ACTH) is a hormone produced by the pituitary gland that binds
271 its receptor in the cells of the zona fasciculata of the adrenal cortex and drives the production
272 of corticosterone. Elevated circulating corticosterone levels induce negative feedback on the
273 hypothalamus and inhibit ACTH release. In order to assess whether the impaired synthesis of
274 corticosterone in tumor-bearing mice fed KD is a) a localized phenomenon in the cortex of the
275 adrenal glands, b) due to an upstream defect in the HPA axis, such as inadequate ACTH
276 production by the pituitary gland, or c) a combination of both, we quantified ACTH in the
277 plasma of cachectic mice and littermate controls fed NF or KD. At endpoint, cachectic tumor-
278 bearing mice fed KD showed significantly higher levels of plasma ACTH compared to tumor-
279 bearing mice fed NF, supporting the hypothesis of an intrinsic deficiency in corticosterone
280 biosynthesis in the adrenal glands (Figure 3F). However, given the variance in ACTH levels, a
281 minor contribution from upstream mechanisms to the observed hypocorticosteronemia cannot
282 be ruled out.

283 Since corticosterone release can potentially be driven by direct stimulation of the
284 adrenal glands by non-ACTH peptides such as IL-6 (Bethin, Vogt, and Muglia 2000; Salas et al.
285 1990; Žarković et al. 2008), and the C26 model is known to display high IL-6 levels (Flint et al.
286 2016), we quantified circulating levels of this cytokine. No diet-mediated differences between
287 the tumor-bearing groups were observed (Figure S3D), indicating that IL-6 does not contribute
288 to the relative corticosterone deficiency observed in KD-fed tumor-bearing mice.

289 To asses adrenal gland responsiveness at the onset of cachexia, we performed an ACTH
290 stimulation experiment using synthetic ACTH (synacthen test). Baseline levels pre-stimulation
291 were significantly elevated in tumor-bearing C26 mice on NF compared to tumor-bearing C26
292 mice on KD (Figure 3G). Upon ACTH injection, plasma corticosterone concentration increased
293 over time in tumor-bearing C26 mice fed NF and littermates, while levels in tumor-bearing C26
294 mice fed KD did not significantly change. After 60 minutes, levels of corticosterone in tumor-
295 bearing C26 mice fed NF were almost 2.5-fold higher than in tumor-bearing C26 mice fed KD.
296 Both littermate groups showed similar responses and reached peak levels comparable to those
297 of tumor-bearing C26 mice on NF at baseline (Figure 3G). These data point towards an intrinsic
298 difficulty in the adrenal glands of KD-fed tumor-bearing mice to respond to hormonal
299 stimulation and release corticosterone compared to NF-fed tumor-bearing mice. The same
300 synacthen test was performed in a different mouse cohort four days after enrolment and diet
301 change (day18 post-C26 injection). Both tumor-bearing C26 groups had higher baseline
302 corticosterone levels compared to the littermate controls. In response to ACTH administration,
303 tumor-bearing C26 mice on NF had a stronger response to ACTH than their littermate controls.
304 Conversely, corticosterone upregulation in tumor-bearing C26 mice on KD was significantly
305 reduced compared to the response of control mice on the same diet (Figure 3H). Thus, our
306 results indicate early signs of malfunction of the stress axis in tumor-bearing mice fed KD even
307 only 4 days after dietary change, but these only become evident and clinically relevant at the
308 onset of cachexia, when an adequate stress response that coordinates adaptation and systemic
309 homeostasis is essential. Altogether these data provide evidence that KD drives the
310 development of a relative hypocorticosteronemia in tumor-bearing C26 mice fed KD compared
311 to those fed NF.

312

313 **NAC treatment rescues corticosterone synthesis in tumor-bearing mice fed ketogenic diet**

314 In order to identify the mechanism underlying the relative deficiency in corticosterone
315 biosynthesis in tumor-bearing mice fed KD, we next explored the interaction of the GSH
316 pathway (Figure S2A) and the corticosterone synthesis pathway (Figure S3A) through their
317 common need of NADPH sources. Targeted quantification of NADPH in the adrenal glands of

318 cachectic mice and controls showed increased levels of NADPH in tumor-bearing C26 mice fed
319 NF, as it would be anticipated in the context of an ongoing release of corticosterone. However,
320 the NADPH supply was diminished in the adrenal glands of tumor-bearing C26 mice fed KD.
321 Administration of NAC, a cysteine prodrug that replenishes intracellular GSH levels in the
322 absence of NADPH consumption, rescued NADPH levels in these mice (Figure 4A). Taken
323 together, these findings indicate that the increased demand for NADPH in the process of
324 detoxification of LPPs leads to a shortage of this cofactor molecule, which then is not available
325 for use in the synthesis of corticosterone and leads to low levels of this stress hormone in
326 tumor-bearing mice fed KD.

327 To examine this hypothesis further, we measured corticosterone levels in tumor-bearing
328 mice fed KD or NF and treated with NAC. Circulating corticosterone was markedly higher in the
329 NAC-treated groups compared to untreated and control groups on the same diet (Figure 4B).
330 Simultaneously, pregnenolone accumulation in KD-fed tumor-bearing mice was no longer
331 detected upon NAC treatment (Figure 4C) indicating conversion of this early intermediate to
332 downstream intermediates of corticosterone biosynthesis and ultimately to corticosterone.
333 Therefore, promoting GSH production through NAC diminishes the need of NADPH oxidation,
334 consequently increasing GSH's LPP-detoxifying activity and at the same time preventing NADPH
335 depletion. NADPH availability enables an appropriate synthesis of corticosterone in the
336 adrenals and leads to the physiological rise in systemic corticosterone levels in the context of
337 metabolic stress associated with cachexia.

338

339 **LPPs exposure decreases cortisol production in a human adrenal cortex-derived cell line**

340 We next implemented the human adrenal cortex-derived cell line, H295R, to test the
341 direct effects of LPPs on cortisol synthesis *in vitro*. We first identified for 4-hydroxynonenal (4-
342 HNE), 4-hydroxyhexenal (4-HHE) and malondialdehyde (MDA) tolerated doses that did not
343 affect cell viability as assessed by Sulforhodamine B (SRB) survival assays, a widely used method
344 for *in vitro* cytotoxicity screening. After exposing the cell line to tolerated doses of 4-HNE
345 (Figure 4D), we quantified the levels of cortisol released to the media at 48h and 72h after
346 exposure to 4-HNE, in untreated cells, or in cells that were exposed to 5 µM 4-HNE once daily.

347 The results show significantly diminished cortisol concentration upon daily exposure to 5 μM 4-
348 HNE, as well as reduced cortisol levels after 72h of exposing the cells to a single-dose of 3 μM or
349 5 μM 4-HNE (Figure 4G). Similarly, treatment of H295R cells with a tolerated dose of 4-HHE led
350 to lower cortisol production compared to untreated adrenocortical cells (Figure 4E and 4H).
351 This cortisol-suppressing effect was even more robust upon treatment of H295R cells with a
352 tolerated dose of MDA (Figure 4F). Single exposure to 150 μM MDA led to a 6-fold decrease in
353 cortisol production by H295R adrenocortical cells after 48h, and daily treatment did not amplify
354 the inhibition compared to single treatment (Figure 4I). Thus, these data suggests that LPPs
355 exert a direct effect on cortisol production in adrenocortical human cells *in vitro*.
356

357 **GDF-15 is elevated in cachexia and increased by ketogenic diet**

358 While the KD-mediated biochemical impairment of the adrenal glands stress response
359 and resulting defective glucocorticoid biosynthesis in tumor-bearing mice described above can
360 account for shortened survival, it does not necessarily explain reduction in food intake. GDF-15,
361 a TGF-beta superfamily member that has been shown to induce reduced food intake by binding
362 its cognate receptor GFRAL in the area postrema, is produced by cells under stress including
363 metabolic stress (Patel et al. 2019). It has been implicated in the anorectic response in
364 cancer cachexia (Hsu et al. 2017). In another model of aldehyde toxicity-induced anorexia, GDF-
365 15 reversibly modulated food intake (Mulderig et al. 2021). In keeping with these findings, we
366 observed elevated circulating GDF-15 levels in the C26 model system during cachexia in NF-fed
367 mice, which were further elevated in KD-fed cachectic mice (Figure 5A), reflecting the systemic
368 oxidative and metabolic stress of the organism and explaining at least in part the reduced food
369 intake observed in the cachectic phase of the disease progression (Figure S1F and S1G).
370

371 **Appropriate usage of energy sources in the context of cachexia is impaired in ketogenic diet- 372 fed tumor-bearing mice**

373 To test the relevance of glucocorticoid-driven metabolic adaptation that promotes
374 survival in the context of a cachectic tumor-bearing host and the impact that its malfunction

375 may have in the context of KD feeding, we assessed and compared the systemic metabolic state
376 of these mice.

377 At endpoint, tumor-bearing mice in both dietary groups exhibited signs of wasting and
378 cachexia, including splenomegaly, and loss of fat and muscle mass (Figure S1D and S1E).
379 Atrogin-1 and MuRF1 are markers of skeletal muscle atrophy and proteolysis of muscle proteins
380 in various pathological conditions, including cachexia (Yuan et al. 2015). mRNA Quantification of
381 these two muscle-specific E3 ubiquitin ligases in the quadriceps of cachectic mice exhibited
382 upregulated expression in both tumor-bearing C26 groups compared to controls, yet the fold
383 increase was significantly less pronounced in tumor-bearing KD fed than in NF fed mice (Figures
384 5B and 5C). Creatinine, an end product of muscle catabolism, was markedly increased in the
385 circulation of cachectic tumor-bearing C26 and KPC mice fed NF but not in in cachectic tumor-
386 bearing mice fed KD (Figures 5D and 5E), presumably as a consequence of lower corticosterone
387 levels that regulate the breakdown of proteins. Urea production is commonly upregulated in
388 cachexia due to increased protein release from muscle tissue breakdown (Corbello Pereira et al.
389 2004; Haines et al. 2019), however, urea levels stayed low in cachectic tumor-bearing mice fed
390 KD compared to cachectic tumor-bearing mice on NF (Figures S4A and S4B). These observations
391 indicate that, in cachectic tumor-bearing mice fed KD, the process of ubiquitin-mediated
392 proteolysis is impaired despite exhibiting cachexia-associated muscle atrophy.

393 We next examined how the liver responded to the metabolic stress in cachectic tumor-
394 bearing mice undergoing systemic wasting and decreased food intake. RNAseq data from livers
395 of cachectic tumor-bearing KPC mice fed NF or KD demonstrated downregulated hepatic
396 glycolysis, glucose metabolism and pyruvate metabolism in KD-fed KPC, all of which are
397 pathways that are usually stimulated by glucocorticoids (Figure 5F). Peroxisomal lipid
398 metabolism and fatty acid metabolism appeared upregulated in the liver of tumor-bearing KPC
399 mice fed KD compared to those fed NF, in agreement with a predominantly lipid-rich diet
400 (Figure 5G). Moreover, untargeted metabolomics of the liver of littermate controls and tumor-
401 bearing C26 mice on either KD or NF diets manifested distinct hepatic metabolic profiles
402 between the groups (Figure S4C). Specific pathway analysis led us to identify a marked
403 accumulation of fatty acid metabolites in the liver of tumor-bearing C26 mice fed KD compared

404 to those fed NF (Figure 5H). These data, together with the observation of macroscopically
405 bigger liver sizes (Figure S1D), suggested that inappropriate macronutrient utilization of lipids
406 could account for the inability to sustain glucose homeostasis and the build-up of unprocessed
407 fat in the liver of tumor-bearing mice on KD. In addition, tricarboxylic acid (TCA) cycle metabolic
408 intermediates appeared clearly upregulated in the liver of tumor-bearing C26 mice fed NF
409 compared to C26 mice fed KD, which were unable to increase these metabolites to the same
410 extent (Figure 5H). The Krebs or TCA cycle in hepatic cells is the metabolic progenitor pathway
411 for gluconeogenesis and the main source of energy for the body (Anderson et al. 2018; Rui
412 2014). Glycerol and aminoacids, such as glutamine and arginine, are a major cellular carbon
413 source for oxidative catabolism via the TCA cycle. These energy substrates were elevated in the
414 liver of tumor-bearing C26 mice fed KD (Figure 5I, 5J and 5K), reinforcing the idea of an
415 impaired host metabolic activity and deficient energy production to sustain survival in these
416 mice.

417 Altogether, these data provide evidence that the lack of an appropriate corticosterone
418 release in tumor-bearing mice fed KD is associated with metabolic maladaptation, inability to
419 use energy sources, failure to achieve glucose homeostasis, and ultimately earlier onset of
420 cancer cachexia.

421

422 **Dexamethasone treatment extends survival and improves metabolic adaptation of tumor- 423 bearing mice fed ketogenic diet**

424 Given the relevance of the steroid hormone corticosterone in metabolic adaptation and
425 efficiency of energy utilization under stress conditions such as cancer cachexia, we next tested
426 the hypothesis that the survival disadvantage in tumor-bearing mice fed KD compared to NF fed
427 is driven by the differences in corticosterone production.

428 BALB/c mice bearing subcutaneous C26 colorectal tumors for 14 days were either fed
429 KD or NF and treated daily with 1mg/kg dose of Dexamethasone intraperitoneally.
430 Dexamethasone is a synthetic long-acting potent glucocorticoid analogue, with a glucocorticoid
431 activity of 30 relative to cortisol (Liu et al. 2013; Mager et al. 2003). Treatment with
432 Dexamethasone extended survival in tumor-bearing C26 mice fed KD compared to all other

433 tumor-bearing groups fed with either diet. Specifically, Dexamethasone led to a delay in the
434 onset of cachexia and a major improvement in overall survival (OS) of C26 mice fed KD
435 compared to the untreated group (Median OS: 10 days C26/KD, 33 days C26/KD + Dex).
436 Dexamethasone treatment of NF fed tumor-bearing C26 mice also significantly extended overall
437 survival (OS) (endpoint defined as >15% bodyweight loss) for 5 days (Median OS: 14 days
438 C26/NF, 19 days C26/NF + Dex), but to a lower degree than on KD fed C26 mice (Figure 6A,
439 Figure S5B). Moreover, Dexamethasone treatment prompted faster growth of tumors in C26
440 mice fed NF and therefore shortened progression free survival (PFS) (endpoint defined as tumor
441 size > 2000 mm³) in these mice (Figures 6B and S5C). Tumor sizes in C26 mice fed KD were not
442 significantly affected by Dexamethasone treatment and tumor weight remained unchanged,
443 whereas tumors in Dex-treated C26 mice fed NF were increased almost 2-fold at endpoint
444 (Figure 6C).

445 After 4 days of treatment with Dexamethasone, the depletion of fat stores induced by
446 KD in tumor-bearing animals was rescued, but fat tissues in C26 mice fed NF showed no
447 differences in weight upon Dexamethasone treatment (Figure 6D). This is in agreement with
448 hepatic metabolomics data at endpoint, which shows decreased fatty acid metabolism in the
449 liver of KD-fed C26 tumor-bearing mice treated with Dexamethasone compared to non-treated
450 C26 tumor-bearing mice on KD. No changes in fatty acid metabolism were detected in NF-fed
451 mice on Dexamethasone (Figure S5D). Quadriceps and liver mass were not affected by
452 Dexamethasone in any of the groups, and splenic size was reduced in both C26 tumor-bearing
453 groups, but more significantly in KD fed mice, as a consequence of the immunosuppressive
454 effects of Dexamethasone (Figure S5E).

455 To investigate further the metabolic changes induced by corticosteroids during cancer
456 progression and cancer cachexia in tumor-bearing mice on NF and KD diets, we next placed KD
457 and NF fed tumor-bearing C26 mice, untreated or treated daily with 1mg/kg dose of
458 Dexamethasone, and controls, in metabolic cages capable of precise monitoring of multiple
459 metabolic parameters (details in Methods). The Respiratory Exchange Ratio (RER) is a ratio
460 between the volume of CO₂ being produced by the body and the amount of O₂ being
461 consumed. A value of RER close to 1.0 depicts the use of carbohydrates as a source of energy,

462 whereas a ratio of 0.7 is indicative of fatty acids used as the primary fuel (Bhandarkar et al.
463 2021). RER values of littermate controls on NF fluctuated from carbohydrate-focused during
464 night hours, when mice are physically more active and eating, to a more mixed energy source
465 during light hours, when mice tend to sleep (Figure 6E). Littermate controls on KD exhibited
466 moderate circadian rhythms in RER too, but values always depicted fat consumption as energy
467 source. At the onset of cachexia, RER values in tumor-bearing C26 mice fed NF flattened
468 compared to controls on the same diet, indicating that caloric restriction associated with
469 cachexia induces a switch from carbohydrate utilization to exploitation of other energy sources,
470 such as protein or fat stores, that may be responsible for the depletion of muscle and adipose
471 tissue (Figure 6E). Cachectic tumor-bearing C26 mice on KD were unable to adapt their RER and
472 kept using fat reserves, with extremely low RER values around 0.65 and no diurnal fluctuations
473 (Figure 6E). However, Dexamethasone-treated tumor-bearing C26 mice fed KD exhibited a
474 fluctuating metabolism, with fat used as nutrient fuel during light hours but an increase in RER
475 during night time that suggested improved metabolic adaptation and less utilization of fat as
476 energy fuel (Figure 6E). This is in keeping with the observed fat tissue preservation and reduced
477 fatty acid metabolism in these mice (Figure 6D and S5D). Moreover, Dexamethasone treatment
478 increased circulating glucose levels (Figure 6F), hepatic gluconeogenesis and TCA cycle activity
479 (Figure 6G and 6H) in C26 tumor-bearing mice fed KD, therefore improving glucose-homeostasis
480 and whole-body metabolic adaptation and may ultimately be responsible for the observed
481 extended survival of these mice.

482 Dexamethasone treatment did not change RER levels of C26 mice fed NF, but led to
483 changes in circadian rhythmicity that were almost opposed to littermate controls and untreated
484 C26 mice on NF (Figure 6E). Energy expenditure or heat, referred to the amount of energy (kcal)
485 used to maintain essential body functions per hour, was significantly diminished in cachectic
486 mice compared to their counterparts on the same diet. Dexamethasone treatment markedly
487 raised the energy expenditure of tumor-bearing C26 mice on KD, indicating a beneficial effect of
488 Dexamethasone on the metabolic homeostasis of tumor-bearing mice fed KD, as opposed to
489 C26 tumor-bearing mice fed NF that showed unchanged energy expenditure upon treatment
490 with Dexamethasone (Figure 6I). Dexamethasone treatment also significantly enhanced food

491 and water intake in tumor-bearing C26 mice fed either diet to levels higher than their untreated
492 tumor-bearers (Figures 6J and S5F). This anorexia-blocking effect is independent of the
493 metabolic benefits of Dexamethasone, which are exclusive to KD-fed mice. Tumor-bearing mice
494 minimized their total movement in all axis as they developed cachexia, but Dexamethasone
495 treatment increased the total activity of tumor-bearing C26 mice on KD compared to their
496 untreated counterparts. Dexamethasone did not have an effect on the movement of C26 mice
497 fed NF (Figure S5G).

498 In combination, these results are consistent with a systemic role of glucocorticoids in
499 counteracting cachexia-induced metabolic stress by coordinating adaptive metabolic, feeding
500 and behavioral responses in order to promote survival. Furthermore, the well-known
501 immunosuppressive side effect of glucocorticoid drugs becomes detrimental in the context of a
502 carbohydrate-based diet because it prompts immune escape (Flint et al. 2016) and
503 consequently rapid tumor growth, yet this is overcome in a lipid-enriched, low-carbohydrate
504 diet that induces ferroptotic death of cancer cells. Altogether, this supports a potential
505 synergistical benefit of combining cancer-targeted nutritional interventions with systemic
506 approaches that ameliorate cancer cachexia.

507

508 DISCUSSION

509 In this study, we find that KD administration to murine models bearing interleukin-6
510 associated tumors that reduce hepatic ketogenesis (Flint et al. 2016), uncouples tumor
511 progression from overall survival. We trace this to a biochemical sequence where fat-enriched
512 diets enhance the production of lipid-derived reactive molecules that saturate the GSH pathway
513 and deplete NADPH stores. This induces elevations of the stress-induced suppressant of
514 appetite GDF-15. In addition, insufficiency of NADPH cofactor leads to a biochemically-induced
515 relative hypocorticosteronemia and, consequently, metabolic maladaptation in response to
516 stress and earlier onset of cancer cachexia. Dexamethasone administration improves food
517 intake, systemic metabolism, and ultimately, extends survival of tumor-bearing mice fed KD.
518 The relative adrenal insufficiency may only be subclinical in humans, because our preclinical
519 study shows lack of an appropriate upregulation of cortisol in the context of metabolic stress,

520 not a complete absence of the hormone. Thus, even if not presenting apparent symptoms, HPA
521 axis activity and adrenal gland responsiveness should be assessed in patients with a lipid-rich
522 nutritional intake, since this will have effects in systemic metabolism and therapeutic outcome.

523 These findings caution against a universal utilization of delayed tumor growth as a
524 predictor of prolonged overall survival in cancer models and in the clinic. They highlight that the
525 homeostatic control of energy balance is a highly evolved biological process that involves the
526 coordinated regulation of food intake and energy expenditure. Disruption in metabolic
527 homeostasis causes poor prognosis and early death of patients with cancer (Hursting and
528 Berger 2010), suggesting that therapeutic support of host metabolic adaptation may extend
529 lifespan. Indeed, we find that rescue of this systemic metabolic adaptation (KD plus
530 Dexamethasone) suppresses cancer cachexia and extends survival without altering tumor
531 burden, indicating that it is the systemic metabolic imbalance that is most lethal. At the same
532 time, targeting metabolic dependency of cancer cells shows therapeutic promise, in that it can
533 stall or delay tumor growth. However, our results show that careful consideration of this
534 paradigm is indicated, if a chosen nutritional intervention challenges both the organism and the
535 cancer cell metabolism. This is specifically the case for ketogenic diet interventions, which are
536 currently tested in clinical trials. Here, the anti-cancer effect may be offset by the inability of
537 the organism to utilize the fatty acid nutrients, because reprogramming of systemic
538 metabolism, muscle and fat loss, and reduced food intake are hallmarks of organisms with
539 cancer progression and cancer cachexia (Janowitz 2018).

540 Glucocorticoids, and specifically cortisol regulate metabolism in conditions of stress.
541 Cortisol increases the availability of blood glucose to the brain and stimulates fat and
542 carbohydrate metabolism. Dexamethasone is a corticosteroid commonly used as a supportive
543 care co-medication for patients with cancer undergoing standard care in order to lower the
544 immune response and reduce inflammation. It is also used as first-line single agent or in
545 combination to prevent or treat cancer-related conditions such as anemia, cerebral oedema,
546 hypersensitivity, hypercalcemia and thrombocytopenia. Side effects of Dexamethasone
547 treatment include weight gain, increased glucose levels and fat accumulation. However, our
548 study shows that what would normally be considered in the clinic as metabolic aftereffects of

549 Dexamethasone may become beneficial for pre-cachectic organisms on a fat-rich diet that
550 exhibit metabolic imbalance and inadequate response to preserve glucose homeostasis (e.g.,
551 HPA axis unresponsiveness).

552 Moreover, some preclinical studies and clinical trials suggest that corticosteroid-induced
553 immunosuppression might dampen the activity of cancer chemo-immunotherapy and increase
554 risk of cancer recurrence (Arbour et al. 2018; Eggermont et al. 2020; Fucà et al. 2019), but this
555 is contradicted by others (Massucci et al. 2020; Menzies et al. 2017). This ongoing debate on
556 the potential impact of corticosteroids on the anti-tumor immune response is illustrated in our
557 preclinical work, as we observe that Dexamethasone administration reduces PFS in mice fed NF
558 while tumor growth is unaffected by Dexamethasone treatment in mice fed KD. Thus, the
559 patients' dietary intake and nutritional state may be cofounding factors in clinical trials that
560 investigate the immunosuppressive impact of corticosteroids co-treatment in cancer.
561 Nutritional interventions combined with the minimum effective dose should be preferred in the
562 context of corticosteroid treatment.

563 Dietary interventions can be used to enhance anticancer therapy and improve clinical
564 outcomes (de Groot et al. 2020; Hopkins et al. 2018; Maddocks et al. 2017). Cancer cells exhibit
565 a high rate of glycolysis in the presence of ample oxygen, a process termed aerobic glycolysis
566 (Warburg effect) (Warburg 1925), and this glucose-dependency of tumors can be exploited by
567 specific diet regimens depleted of the nutrients that tumors use as energy source. Diets can
568 also have an effect on the immune system and influence the anti-tumor response. KDs have
569 been previously studied in patients with cancer, and they have been shown to be safe, feasible,
570 and even to have anticancer effects. These low-carbohydrate diets reduce circulating glucose
571 levels and therefore, successfully starve tumors. In this study, we show evidence that supports
572 that KD may be slowing down tumor growth not only via glucose deprivation of the tumor but
573 also through an ongoing accumulation of non-detoxified highly-reactive lipid peroxidation
574 products that causes ferroptotic cell death within the tumor. KD's aminoacid composition may
575 also be contributing to the observed ferroptosis and metabolic phenotype, as the 9-fold
576 decrease in cystine in the nutritional profile of KD compared to NF can be linked to an induction
577 of ferroptosis independently of fatty acids (Badgley et al. 2020). Changes in metabolism and

578 energy expenditure in KD-fed mice could be partially attributed to the 3-fold decrease in
579 methionine intake (Pissios et al. 2013) (Extended Data Table 1).

580 We note that the effect of KD on the host is likely context-dependent: it is detrimental
581 for both the tumor and the host that has been metabolically reprogrammed and bears an
582 established tumor, uncoupling tumor size from overall survival; but KD has no impact on non-
583 tumor-bearing hosts that are able to adapt their metabolism to the nutritional intake. Even if
584 they grow a tumor later on, the metabolic reprogramming induced by the tumor is delayed due
585 to its decelerated growth. This explains differential results in the literature (Lien et al. 2021) and
586 evidences the importance of dosing and timing in the clinic.

587 Without independent expansion and validation of our results, it is perhaps too early to
588 suggest that KD and glucocorticoid co-administration may be a therapeutic strategy for patients
589 with interleukin-6 elevating cancers. Nevertheless, the comparatively reduced tumor
590 progression and prolonged overall survival in mice on KD and Dexamethasone compared to
591 mice on NF with or without Dexamethasone is an encouraging finding. A limitation to this
592 concept and to our study is a lack of the exact understanding of how glucocorticoids reprogram
593 and rescue metabolism in the context of a reprogrammed organism challenged with high lipid
594 diet. While this may limit the ability for longitudinal molecular response monitoring of patients
595 on the combination intervention, weight trajectories and glucose levels may be suitable and
596 readily obtainable biomarkers for clinical studies.

597

598 CONCLUSION

599 Our study highlights that the effect of systemic interventions cannot necessarily be
600 extrapolated from the effect on the tumor alone, but that they have to be investigated for anti-
601 cancer and host effects. In model systems with established tumors that elevate interleukin 6,
602 the opposing effect of a KD nutrition on delayed tumor growth and induction of cachexia, lead
603 to a dominant negative effect on overall survival. These findings may be relevant to clinical
604 research efforts that investigate the potential benefit of KD for patients with cancer.

605

606 LIMITATIONS

607 Our results have been obtained using model systems of colorectal and pancreatic cancer
608 that are known to recapitulate clinical disease progression from early cancer to cancer cachexia,
609 but clinical validation of our work is needed. We acknowledge that not all cancers lead to IL-6
610 elevations and we cannot comment on the transferability of our findings to cancers that are not
611 associated with raised IL-6. The time-course of disease progression and metabolic
612 reprogramming in patients with cachexia inducing tumors has not been resolved with high
613 resolution. We here focus on mice with fully established tumors that are challenged with KD
614 when early metabolic reprogramming has occurred. Future studies have to guide how
615 preclinical work like this is best translated to clinical cancer progression and how this alignment
616 could guide stratified enrolment of patients into interventional trials. Our work also provides no
617 definitive answer on dosing for glucocorticoid replacement and, here too, detailed clinical
618 studies are required to define the best therapeutic dose range.

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631

632 AUTHOR CONTRIBUTIONS

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634 E.E.D., S.O.K., M.Z., J.H, R.R., T.R.F., C.M.C., and M.L.; Writing – Original Draft, M.F.; Writing –
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637

638 DECLARATION OF INTERESTS

639 The authors declare no competing interests.

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649 FIGURE TITLES AND LEGENDS

650 **Figure 1. Ketogenic diet slows down tumor growth but shortens overall survival in C26 and**
651 **KPC murine models of cancer cachexia.** (A) Longitudinal tumor volume in C26 tumor-bearing
652 mice fed ketogenic (KD) or standard (NF) diets (n=12). (B) Longitudinal tumor area in KPC
653 tumor-bearing mice fed KD or NF (n=8). (C) Overall survival of C26 tumor-bearing mice and
654 littermate controls on KD or NF (n=7 LM, n=17-18 C26). (D) Overall survival of KPC tumor-
655 bearing mice and PC controls fed KD or NF (n=6-8). (E-F) Longitudinal glucose measurements
656 from day 0 of diet change to cachectic endpoint in C26 tumor-bearing mice and littermate
657 controls (n=5 LM, n=20 C26) (E), and in KPC tumor-bearing mice and PC controls (n=10) (F), fed
658 either KD or NF diets. (G-H) Longitudinal ketone measurements from day 0 of diet change of
659 cachectic endpoint in C26 tumor-bearing mice and littermate controls (n=5 LM, n=20 C26) (G),
660 and in KPC tumor-bearing mice and PC controls (n=10) (H), fed either KD or NF diets.

661 Data are expressed as the mean \pm SEM. Overall survival (OS): time until mice reach >15%
662 bodyweight loss. Differences in (A-B) were assessed by fitting a mixed effect model with
663 coefficients for the intercept, slope and the difference in the slope between diets, and a
664 random component for each individual mouse. Kaplan–Meier curves in (C-D) were statistically
665 analyzed by using the log-rank (Mantel–Cox) test. Two-way ANOVA statistical tests with Tukey’s
666 correction for post hoc comparisons were performed in (E-H). * p-value < 0.05, ** p-value <
667 0.01, *** p-value < 0.001, **** p-value < 0.0001.

668

669 **Figure 2. Ketogenic diet induces ferroptotic cell death of cancer cells that can be prevented by**
670 **NAC.** (A-B) Quantification by UPLC-MS/MS of 4-hydroxynonenal (4-HNE) and GSH/GSSG ratio
671 (B) in the liver of C26-tumor bearing and littermate controls on KD or NF diets (n=7). (C-D)
672 Quantification by UPLC-MS/MS of GSH/GSSG ratio (C) and cysteine (D) in the tumor of C26 mice
673 (n=7). (E) Detection of 4-HNE adducts in tumor lysates from C26 tumor-bearing mice untreated
674 or treated with N-acetyl cysteine (NAC) and fed KD or NF (n=3). (F) 4-HNE adducts formation in
675 tumor of KPC mice fed KD or NF (n=4-6). (G-H) Concentration of ferric (III) and ferrous (II) iron in
676 the tumor of C26 mice fed KD or NF, untreated or treated with NAC intraperitoneally (n=5) (G),

677 and in tumors of KPC mice fed either diet (n=6) (H). (I) Haematoxylin and eosin (H&E) staining of
678 tumors from C26 mice fed KD or NF. (J) Weight of tumors at the time of cachexia in C26 tumor-
679 bearing mice fed KD untreated or treated with NAC (n=7-10). (K) Longitudinal tumor volume of
680 C26 mice fed standard diet and treated with RSL3 or vehicle control (n=4-6). (L) GSEA of
681 upregulated and downregulated pathways in tumors of KPC mice fed KD compared to KPC fed
682 NF (n=5).

683 Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's correction for post hoc
684 testing was used in (A, E-G). Statistical differences in (B-D, H-I) were examined using an
685 unpaired two-tailed Student's t-test with Welch's correction. Simple linear regression model
686 was applied to (J). Statistical analysis in (K) is described in Methods. * p-value < 0.05, ** p-value
687 < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

688

689 **Figure 3. Ketogenic diet induces relative corticosterone deficiency in C26-tumor bearing mice.**
690 (A) Corticosterone hormone levels in plasma of cachectic C26 tumor-bearing mice and
691 littermate controls fed KD or NF diets (n=5 LM, n=10-14 C26). (B-C) Plasma cholesterol levels in
692 cachectic C26-tumor bearing mice and littermate controls (n=5 LM, n=10-11 C26) (B), and in
693 cachectic KPC tumor-bearing mice and PC controls (n=5-8) (C) fed KD or NF diet. (D)
694 Pregnenolone hormone levels in plasma of cachectic C26 tumor-bearing mice and littermate
695 controls on KD or NF diets (n=16-22). (E) Sodium levels in plasma of cachectic C26-tumor
696 bearing mice and littermate controls on KD or NF diets (n=5 LM, n=10-11 C26). (F) Levels of
697 adrenocorticotropic hormone (ACTH) in plasma of cachectic C26 tumor-bearing mice and
698 littermate controls fed KD or NF (n=6-10 LM, n=12-20 C26). (G-H) Synacthen test and
699 quantification of corticosterone response at baseline and 15, 30 and 60 minutes after ACTH
700 stimulation in cachectic C26 tumor-bearing mice and littermate controls (endpoint) (G), or only
701 4 days after diet change (18 days after C26 cell injection) (n=5) (H).

702 Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's correction for post hoc
703 testing was used in (A-D, F). Two-way ANOVA statistical tests with Tukey's correction for post
704 hoc comparisons were performed in (G-H). * p-value < 0.05, ** p-value < 0.01, *** p-value <
705 0.001, **** p-value < 0.0001, # p-value < 0.05 compared to time = 0.

706 **Figure 4. Effect of lipid peroxidation products (LPPs) on adrenal function and rescue with NAC.**

707 (A) NADPH quantification in the adrenal glands of littermate controls and C26 tumor-bearing
708 mice fed KD or NF, and C26 tumor-bearing mice fed KD treated with NAC (n=5 LM, n=9-11 C26).
709 (B-C) Corticosterone (n=5 LM, n=10-14 C26) (B) and pregnenolone (n=5-22) (C) levels in plasma
710 of littermate controls and C26 tumor-bearing mice fed KD or NF, untreated or treated with
711 NAC. (D-F) SRB assays of H295R cells treated for 72h with increasing concentrations of 4-HNE
712 (D), 4-HHE (E) or MDA (F) (n=3 independent experiments). Viability is expressed relative to
713 vehicle-treated control cells. (G-I) Cortisol levels in the media of H295R cells treated with 4-HNE
714 (n=3-6) (G), 4-HHE (n=3-6) and MDA (n=6-15) (I) at 48h and 72h timepoints.

715 Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's correction for post hoc
716 testing was used in (A-C, G-I). * p-value < 0.05, *** p-value < 0.001, **** p-value < 0.0001.

717 **Figure 5. Appropriate usage of energy sources in the context of cachexia is impaired in KD-fed**
718 **tumor-bearing mice.** (A) Levels of GDF-15 in the plasma of C26 tumor-bearing mice and
719 littermate controls fed KD or NF (n=11-19). (B-C) mRNA levels of the E3 ligases Atrogin-1 (B) and
720 MuRF1 (C) in the quadriceps of C26 tumor-bearing mice and littermate controls fed KD or NF
721 (n=5 LM, n=12 C26). (D-E) Plasma creatinine levels in C26 tumor-bearing mice and littermate
722 controls (n=5 LM, n=9-11 C26) (D), and KPC tumor-bearing mice and PC controls (n=5-8) (E) fed
723 either KD or NF. (F-G) GSEA analysis of downregulated (F) and upregulated (G) pathways in KD-
724 fed C26 tumor-bearing mice compared to those NF-fed (n=5). (H) Heatmap of metabolites and
725 specific metabolic pathways in C26 tumor-bearing mice fed KD or NF (n=5). (I-K) Quantification
726 by UPLC-MS/MS of the main substrates of the TCA cycle, glycerol (I), glutamine (J), and arginine
727 (K) in the tumor of C26 mice fed KD or NF (n=7).

728 Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's correction for post hoc
729 testing was used in (A-E). Statistical analysis in (F-G) is described in Methods. Statistical
730 differences in (I-K) were examined using an unpaired two-tailed Student's t-test with Welch's
731 correction. * p-value < 0.05, *** p-value < 0.001, **** p-value < 0.0001.

732

733 **Figure 6. Dexamethasone treatment extends survival and improves metabolic adaptation of**
734 **C26 mice fed ketogenic diet.** (A-B) Overall survival (OS) (A) and Progression Free survival (PFS)
735 (B) of C26 tumor-bearing mice fed KD or NF, untreated or treated with Dexamethasone, and
736 littermate controls fed with either diet (n=7 LM, n=17-18 C26, n=7 C26 + Dex). (C) Weight of
737 tumors in C26 tumor-bearing mice fed KD or NF, untreated or treated with Dexamethasone, at
738 endpoint (n=9-10 C26, n=4-5 C26 + Dex). (D) Quantification of subcutaneous and gonadal fat
739 stores in C26-tumor bearing mice fed KD or NF, untreated or treated with Dexamethasone, 4
740 days after diet change and start of treatment (n=3-10). (E) Cumulative food intake (kcal) during
741 the last 4 days before endpoint in littermate controls and C26 tumor-bearing mice, untreated or
742 treated with Dexamethasone, fed KD or NF (n=7). (F) Plasma glucose levels in C26-tumor
743 bearing mice fed KD or NF, untreated or treated with Dexamethasone, 2 days after diet change
744 and start of treatment. (G-H) Quantification by UPLC-MS/MS of metabolites involved in
745 gluconeogenesis (G) and the TCA cycle (H) in the liver of C26 tumor-bearing mice on either KD
746 or NF diets, untreated or treated with Dexamethasone (n=5). (I-J) Respiratory exchange ratio
747 (RER) (I) and Heat (or energy expenditure) (J) during the last 4 days before endpoint in
748 littermate controls and C26 tumor-bearing mice, untreated or treated with Dexamethasone,
749 fed KD or NF.

750 Data are expressed as the mean \pm SEM. . Overall survival (OS): time until mice reach >15%
751 bodyweight loss. Progression-Free Survival (PFS): time until tumor size reaches > 2000 mm³.
752 Kaplan–Meier curves in (A-B) were statistically analyzed by using the log-rank (Mantel–Cox)
753 test. One-way ANOVA with Tukey’s correction for post hoc testing was used in (C-D, F) Analysis
754 in (E, G-J) is described in Methods. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, ****
755 p-value < 0.0001.

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761 **STAR METHODS**

762 **Laboratory animals**

763 Two different mouse models that predispose to cachexia were used. A transplanted C26
764 model of colorectal cancer and the genetically engineered autochthonous KPC model of
765 pancreatic cancer. The C26 model is performed on wild-type BALB/c mice that are inoculated
766 subcutaneously with a syngeneic tumor. In the KPC system, an activating point mutation (G12D)
767 in Kras and a dominant negative mutation in Trp53 (R172H) are conditionally activated in the
768 pancreas by means of the Cre-Lox technology. Both pre-clinical models have been shown to
769 develop tumors that secrete IL-6 and therefore the host is unable to produce ketones during
770 caloric deficiency associated with cachexia, causing a rise in glucocorticoid levels as a
771 consequence. KPC and BALB/c mice were obtained from Charles River Laboratories. They were
772 kept in pathogen-free conditions on a 24 hour 12:12 light-dark cycle and allowed to acclimatize
773 for 7 days. All animal experiments and animal care at the MRC CU and CRUK CI were performed
774 in accordance with national and institutional guidelines and approved by the UK Home Office,
775 the animal ethics committee of the University of Cambridge. All animal experiments at CSHL
776 were approved by the Institutional Animal Care and Use Committee (IACUC) and were
777 conducted in accordance with the National Institutes of Health Guide for the Care and Use of
778 Laboratory Animals. Body weights, food intake and clinical signs were monitored on a daily
779 basis. Handling was kept to a minimum. Mice were described as cachectic when they reached
780 weight loss of more than 5% from their peak weight and were sacrificed when tumor size
781 exceeded 2 cm length, when weight loss exceeded 15% from peak weight, or when showing
782 clinical signs of discomfort indicative of cachectic endpoint as stated by the Animal Cachexia
783 Score (ACASCO): piloerection, diarrhea or constipation, hunched posture, tremors, and closed
784 eyes (Betancourt et al. 2019). Death was confirmed by cervical dislocation.

785 **Experimental enrolment**

786 Weight-stable, tumor-bearing male KPC mice with tumors of 3-5 mm size and no
787 evidence of obstructive common bowel duct, and their respective weight- and age-
788 matched control counterparts (PC mice) were enrolled in the experiments.

789 Weight-stable wild-type 9-weeks old male BALB/c mice were inoculated with
790 2x10⁶ viable C26 colorectal cancer cells subcutaneously (s.c.) and enrolled on study
791 together with their respective controls.

792 At day of enrolment (day 14 post-injection of C26 cells), mice were stratified in
793 terms of tumor size, body weight and age, singly-housed and randomly allocated into
794 two experimental matched groups fed with different diets: mice were fed with either
795 standard diet (#5053 PicoLab® Rodent Diet 20; LabDiet) or ketogenic diet (KD) (#F3666;
796 Bio-Serv). Ketogenic diet was given in a Petri dish container that was replaced daily due
797 to potential oxidation of the diet.

798 **Endpoint**

799 Overall survival (OS) or Progression Free Survival (PFS) were the final
800 endpoint of the studies.

801 **Overall Survival**

802 Mice were considered to have reached the OS endpoint when their body
803 weight loss exceeded 15% from their peak weight.

804 **Progression Free Survival**

805 Mice were considered to have reached the PFS endpoint when the
806 volume of their tumors exceeded 2000 mm³, as measured by handheld calipers.

807 **Dexamethasone treatment**

808 Dexamethasone 21-phosphate disodium salt (#D1159; Sigma-Aldrich) was
809 dissolved in dH₂O and administered intraperitoneally (i.p.) at 1mg/kg daily.

810 **N-acetyl cysteine (NAC) treatment**

811 N-Acetyl-L-cysteine (#A9165; Sigma-Aldrich) was dissolved in 0.9% NaCl sterile
812 saline solution (#Z1377; Thermo Fisher) and administered intraperitoneally (i.p.) at
813 150mg/kg daily.

814 **RSL3 treatment**

815 RSL3 ((1S,3R)-RSL3) (#HY-100218A; MedChemExpress) was dissolved in 10%
816 Dimethyl Sulfoxide (DMSO) (#12611S; Cell Signaling), 40% Polyethylene glycol 300
817 (PEG300) (#S6704; Selleck Chemicals), 5% Tween-80 and 45% NaCl 0.9% sterile saline

818 solution (#Z1377; Thermo Fisher) and administered intraperitoneally (i.p.) at 5mg/kg
819 daily.

820 **ACTH stimulation test (synacthen test)**

821 ACTH (#HOR-279, ProSpec) was reconstituted in dH₂O and injected
822 intraperitoneally at a dose of 1 ug/g body weight. Tail blood was collected at 0-, 15-, 30-,
823 and 60-min intervals for determination of plasma corticosterone levels. Each group
824 consisted of five animals.

825 **Metabolic cages**

826 The Comprehensive Lab Animal Monitoring System (CLAMS) from Columbus
827 Instruments was used to monitor and quantify multiple metabolic parameters such as activity,
828 weight (g), drinking (mL), food intake (g), sleep, body core temperature and open circuit
829 calorimetry in animal cages that allows precise control over the light/dark cycle. Data from C26-
830 injected or WT BALB/c control mice on standard or ketogenic diet treated with Dexamethasone
831 or vehicle (saline) was collected real-time during an acclimation period (72 hours), a baseline
832 period (72 hours) and during all the experimental timeline (approximately 33 days) through the
833 Oxymax collection software during 10-30 second intervals. Data was exported and analyzed in
834 RStudio.

835 **Tumor size**

836 PDAC tumors in KPC mice were detected via palpation and high-resolution ultrasound
837 imaging (Veo 2100; VisualSonics), and confirmed at necropsy. Tumor growth was monitored
838 by ultrasound scans assessed at multiple angles. Mice were carefully observed for any
839 macroscopic metastases. Maximum cross-sectional area (CSA) and maximum diameter of the
840 tumors were determined for each timepoint. Tumor development in BALB/c mice was spotted
841 via palpation and monitored daily by caliper measurements. Volume of the tumor was
842 calculated as follows: volume (mm³) = [long axis (mm) x short axis (mm)]² / 2.

843 **Blood and plasma measurements**

844 Tail bleeds and terminal cardiac bleeds were taken. Tail vein bleeds were performed
845 using a scalpel via tail venesection without restraint, and terminal bleeds were obtained
846 through exsanguination via cardiac puncture under isoflurane anesthesia. Tail bleeds were

847 immediately analyzed for glucose and ketone concentration measurements using
848 glucose/ketones stripes and gluco-/keto-meters (Freestyle Optium Neo; Abbott laboratories).

849 Plasma samples were collected from tail or terminal cardiac bleeds using heparin-coated
850 hematocrit capillary tubes to avoid coagulation and were processed as follows: centrifuge spin
851 at 14,000 rpm for 5 min at 4°C, snap frozen in liquid nitrogen and stored at -80°C.

852 Corticosterone was quantified from plasma using the International Corticosterone
853 (Human, Rat, Mouse) ELISA (#RE52211; IBL). The sample incubation step from the IBL assay
854 protocol was 3 hours at room temperature (RT) so as to reach displacement equilibrium as
855 determined by preliminary data. IL-6 levels were measured from plasma using the mouse IL-6
856 Quantikine ELISA Kit (#M6000B; R&D Systems).

857 4-hydroxynonenal (4-HNE) measurements in the plasma were quantified using the
858 Universal 4-Hydroxynonenal ELISA Kit (Colorimetric) (#NBP2-66364; Novus Biologicals).

859 Pregnenolone was measured in the plasma using the Pregnenolone ELISA Kit
860 (Colorimetric) (#NBP2-68102; Novus Biologicals).

861 Levels of Adrenocorticotropin hormone (ACTH) in the plasma were measured with the
862 Mouse/Rat ACTH ELISA Kit (#ab263880; Abcam).

863 **Tissue collection**

864 Liver, tumor, spleen, adrenal glands, quadricep muscle, lungs, and gonadal and
865 subcutaneous fat samples were collected and weighed during necropsy dissection.
866 Subsequently, tumor, liver and spleen samples were cut into 3 equal parts, which were either
867 snap frozen in liquid nitrogen, cryo-embedded in OCT, or fixed in 4% neutral buffered
868 formaldehyde for 24 hours at room temperature (RT) before either being transferred to 70%
869 ethanol and later paraffin-embedded (FFPE) for immunohistochemistry processing. All the
870 other organs and tissue samples were immediately snap frozen and stored at -80°C.

871 **Tissue lysis**

872 Snap frozen tissues stored at -80°C were transferred to dishes on wet ice and cut into
873 pieces with a scalpel. Each piece was weighed and placed into 2mL round-bottom Eppendorf
874 tubes pre-loaded with Stainless Steel beads (#69989; Qiagen) on wet ice. Homogenizer tubes
875 were then filled up with lysis buffer (#AA-LYS-16ml; RayBiotech) and supplemented with

876 Protease Inhibitor Cocktail (#AA-PI; Raybiotech) and Phosphatase Inhibitor Cocktail Set I (#AA-
877 PHI-I; RayBiotech). Samples were homogenized in Tissue Lyser II (#85300; Qiagen) for 5 minutes
878 and then lysates were centrifuged at 4°C for 20 minutes at maximum speed. The supernatant
879 was harvested and kept on ice if testing fresh or stored at -80°C.

880 The Bicinchoninic Acid (BCA) Method was used to determine protein concentration in
881 lysates.

882 Ferrous (Fe^{2+}) and Ferric (Fe^{3+}) iron levels in tissue lysates were measured using the
883 Colorimetric Iron Assay Kit (#ab83366; Abcam).

884 Quantification of 4-HNE-protein adducts in lysates was performed using the Lipid
885 Peroxidation (4-HNE) Assay Kit (#ab238538; Abcam).

886 Detection of NADPH levels in the adrenal glands was performed using the
887 NADP/NADPH-Glo™ Bioluminescent Assay (#G9081; Promega).

888 Oil-Red-O staining

889 Fresh frozen tissue sections of 5-10 μm thickness were mounted on slides, air dried for
890 30-60 minutes at RT and fixed in ice cold 10% neutral-buffered formalin (#HT501128-4L; Sigma-
891 Aldrich) for 5-10 minutes. After rinsing in 3 changes of distilled water and air drying for another
892 30-60 minutes, slides were placed in absolute Propylene Glycol (#P4347; Sigma-Aldrich) for 2-5
893 minutes, then stained in pre-warmed Oil Red O solution (#O0625-25G; Sigma-Aldrich) for 8-10
894 minutes in a 60°C oven, differentiated in 85% Propylene Glycol solution for 2-5 minutes and
895 rinsed in 2 changes of distilled water. Slides were then counterstained with Mayer's
896 hematoxylin (#ab245880; Abcam) for 30 seconds, washed thoroughly with distilled water and
897 mounted with aqueous mounting medium.

898 Immunohistochemistry

899 Tissues were fixed in 4% paraformaldehyde (#50-980-495; Thermo Fisher) for 24 h and
900 then embedded in a paraffin wax block. Sectioning with a cryostat, deparaffinization, antigen
901 retrieval and immunohistochemistry for Ki67 (#14-5698-82; Thermo Fisher) was performed by
902 the CRUK CI Histopathology Core using a Leica Bond III autostainer. The slides were scanned on
903 a Leica Aperio AT2 system and subsequently analyzed in a blinded manner. H&E staining was
904 performed by the Histology Facility at Cold Spring Harbor Laboratory.

905 **Metabolomics**

906 Global metabolic profiling of liver and tumor samples was performed by UPLC-MS/MS at
907 Metabolon, Inc. facilities (UK project #CRUK-01-19VW; USA project #CSHL-01-22VW+; results
908 from both datasets were merged). Samples were prepared using the automated MicroLab
909 STAR® system from Hamilton Company. Several recovery standards were added prior to the
910 first step in the extraction process for QC purposes. To remove protein, dissociate small
911 molecules bound to protein or trapped in the precipitated protein matrix, and to recover
912 chemically diverse metabolites, proteins were precipitated with methanol under vigorous
913 shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting
914 extract was divided into five fractions: two for analysis by two separate reverse phases
915 (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for
916 analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS
917 with negative ion mode ESI, and one sample was reserved for backup. Samples were placed
918 briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were
919 stored overnight under nitrogen before preparation for analysis.

920 Raw data were extracted, peak-identified and QC processed using Metabolon's
921 hardware and software. These systems are built on a web-service platform utilizing Microsoft's
922 .NET technologies, which run on high-performance application servers and fiber-channel
923 storage arrays in clusters to provide active failover and load-balancing. Compounds were
924 identified by comparison to library entries of purified standards or recurrent unknown entities.
925 Metabolon maintains a library based on authenticated standards that contains the retention
926 time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS
927 spectral data) on all molecules present in the library. Furthermore, biochemical identifications
928 are based on three criteria: retention index within a narrow RI window of the proposed
929 identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and
930 reverse scores between the experimental data and authentic standards.

931 A total of 685 and 669 named metabolites were retained for liver and tumor datasets,
932 respectively. Following log transformation and imputation of missing values, if any, with the
933 minimum observed value for each compound, Welch's two-sample t-test was used to identify

934 biochemicals that differed significantly between experimental groups. An estimate of the false
935 discovery rate (q-value) was calculated to take into account the multiple comparisons that
936 normally occur in metabolomic-based studies.

937 **Cell lines**

938 **C26 murine colorectal cancer cell line**

939 C26 cells were cultured in complete growth medium consisting of RPMI-1640
940 medium with Glutamine (#11-875-093; Thermo Fisher) containing 10% of Heat-
941 Inactivated Fetal Bovine Serum (FBS) (#10-438-026; Thermo Fisher) and 1x Penicillin-
942 Streptomycin solution (#15-140-122; Thermo Fisher) under sterile conditions. 1x
943 Trypsin-EDTA (#15400054; Thermo Fisher) was used for cell dissociation. Cells were
944 resuspended in FBS-free RPMI and viable cells were counted using a Vi-Cell counter
945 prior to subcutaneous injection of 2×10^6 viable cells diluted in 100 μ L RPMI into the right
946 flank of each BALB/c mouse.

947 **H295R human adrenocortical cell line**

948 H295R cells (#CRL-2128; ATCC) were cultured in complete growth medium
949 consisting of DMEM:F12 medium (#30-2006; ATCC) with 0.00625 mg/mL insulin;
950 0.00625 mg/mL transferrin; 6.25 ng/mL selenium; 1.25 mg/mL bovine serum albumin;
951 0.00535 mg/mL linoleic acid (ITS+ Premix)(#354352; Corning) and adjusted to a final
952 concentration of 2.5% Nu-Serum I (#355100; Corning). Cells were grown in 75 cm²
953 culture flasks and subcultured 1:3 every 3 days. 1x Trypsin-EDTA solution was used for
954 cell dissociation prior to seeding cells in 96-well plates for experimental viability or
955 cortisol release tests.

956 **H295R Cortisol synthesis assay**

957 H295R cells were seeded in 96-well plates with 12,000 cells in 200 μ L of complete
958 growth medium for each well and allowed to settle for 48 hours. The medium was then
959 changed and 200 μ L medium containing specific LPPs was added. LPPs used: 4-hydroxynonenal
960 (4-HNE) (#32100; Cayman Chemical), 4-hydroxyhexenal (4-HHE) (#32060; Cayman Chemical)
961 and malondialdehyde (MDA) (#63287-1G-F; Sigma-Aldrich). After 48 or 72 hours of incubation
962 in the presence of the compound, the medium was carefully collected, transferred to the test

963 tubes and immediately stored at -20°C. Cells in each well were detached and counted by Trypan
964 Blue staining (#15250061; Thermo Fisher). Samples were diluted 1:5 and cortisol levels were
965 measured using a Cortisol Competitive Human ELISA Kit (#EIAHCOR; Thermo Fisher) according
966 to the manufacturer's protocol.

967 **Sulforhodamine B (SRB) colorimetric assay**

968 A total of 12,000 cells per well were seeded in a 96-wells plate. Two days after seeding,
969 media was replaced and cells were treated with increasing concentrations of a specific LPP for
970 72h. Then, cells were fixed with 1% trichloroacetic acid (#T9159-100G; Sigma-Aldrich) at 4°C
971 for 30 min, washed 3 times with distilled water and stained with 0.057% SRB (#S1402-5G;
972 Sigma-Aldrich) in 1% acetic acid (#A6283, Sigma-Aldrich) solution at RT for 30 min. Following
973 staining, cells were washed 3 times with 1% acetic acid and air-dried overnight. The protein
974 bound dye was dissolved in 10mM Tris base solution and the absorbance was measured at
975 565nm using a microplate reader (SpectraMax i3x).

976 **Single cell preparation**

977 Cell suspensions were prepared from tissues by mechanical dissociation, followed by
978 digestion in 5 mL of RPMI-1640 containing collagenase I (500 U/mL) (#SCR103; Sigma-Aldrich)
979 and DNase I (0.2 mg/mL) (#04716728001; Sigma-Aldrich) for 45 min at 37°C on a shaker (220
980 rpm), followed by filtration through a 70-µm strainer and 25% Percoll (#GE17-0891-01; Sigma-
981 Aldrich) gradient enrichment of leukocytes, and red blood cell (RBC) lysis. Tumor cells were
982 recovered without Percoll enrichment. Blood cells were lysed in 5 mL of RBC lysis buffer
983 (#A1049201; Thermo Fisher) three times for 5 min, and spleens were strained through a 70-µm
984 filter in RPMI-1640 before lysing erythrocytes with RBC lysis buffer for 5 min. Single cells were
985 restimulated and stained for surface and intracellular markers (see flow cytometry below).

986 **Flow cytometry**

987 Cell sorting was performed using a FACSaria™ Cell Sorter (BD Biosciences) at CSHL Flow
988 Cytometry Facility. FlowJo X (Tree Star) software was used for experimental analysis.

989 The following antibodies were used: Alexa Fluor 700 anti-mouse CD45 (#103127;
990 BioLegend), FITC anti-mouse CD45 (#11-0451; Thermo Fisher), APC/Cy7 anti-mouse CD3ε
991 (#100329; BioLegend), PerCP/Cyanine 5.5 anti-mouse CD4 (#100433; BioLegend), Brilliant Violet

992 510TM anti-mouse CD8a (#100751; BioLegend), Brilliant Violet 605TM anti-mouse/human
993 CD11b (#101257; BioLegend), Alexa Fluor 700 anti-mouse Ly-6G/Ly-6C (Gr-1) (#108421;
994 BioLegend), FITC anti-mouse CD69 (#104505; BioLegend), PE/Cy7 anti-mouse CD152 (#106313;
995 BioLegend); Brilliant Violet 421TM anti-mouse CD274 (#124315; BioLegend), PE/Dazzle 594
996 anti-mouse CD279 (#109115; BioLegend), and FITC anti-mouse F4/80 (#123107; BioLegend).

997 **Western blotting**

998 Cells were lysed in RIPA buffer (50mM Tris HCl, pH 7.4, 150mM NaCl, 0.5%
999 deoxycholate, 0.1% sodium dodecyl sulphate, 1% NP-40) (#89901; Thermo Fisher) containing
1000 protease inhibitors (#78442; Thermo Fisher) and 1 mM dithiothreitol (DTT) (#A39255; Thermo
1001 Fisher). Whole cell extracts were separated by electrophoresis, transferred onto nitrocellulose
1002 membranes (#88025; Thermo Fisher) and blocked in 5% non-fat dry milk (#1706404; Bio-Rad)
1003 dissolved in 0.1% Tween/TBS. Membranes were incubated with primary antibodies: BAX Rabbit
1004 mAb (#50599-2-Ig; Proteintech, 1:500), β-Actin Rabbit mAb (#4967; Cell Signaling Technology,
1005 1:5000) and Caspase-3 (D3R6Y) Rabbit mAb (#14220; Cell Signaling, 1:1000), overnight at 4°C
1006 followed by washing in 0.1% Tween/TBS. Membranes were incubated with Goat Anti-Rabbit IgG
1007 H&L (HRP) secondary antibodies (#ab205718; Abcam, 1:5000) at 25°C for 1h and washed thrice
1008 prior to signal detection. Membranes were developed by exposure in a dark room through
1009 chemiluminescence using ECL reagent (#32106; Thermo Fisher).

1010 **qRT-PCR**

1011 mRNA was extracted from frozen tissues using QIAzol Lysis Reagent (#79306; Qiagen)
1012 and the Tissue Lyser II (#85300; Qiagen), following the manufacturer's protocol for the RNeasy
1013 Lipid Tissue Mini Kit (#74804, Qiagen) in an automated manner with the QIAcube Connect
1014 (#9002864; Qiagen). Concentration and purity of aqueous RNA was assessed using a
1015 NanoDrop™ Spectrophotometer (#ND-ONE-W; Thermo Fisher). mRNA templates from muscle
1016 and liver samples were diluted to 2ng/μl and mRNA was analysed by quantitative Real-Time
1017 PCR using the TaqMan™ RNA-to-CT™ 1-Step Kit (#4392653; Thermo Fisher). mRNA levels were
1018 normalized to either Rn18s (liver) or Tbp (quadriceps) using the ddCt method. The following
1019 TaqMan primers were used: Mm01277044_m1 (Tbp); Mm03928990_g1 (Rn18s);

1020 Mm00440939_m1 (Ppara); Mm01323360_g1 (Acadm); Mm00550050_m1 (Hmgcs2);
1021 Mm00499523_m1 (Fbxo32); and Mm01185221_m1 (Trim63).

1022 **RNA-sequencing**

1023 RNA extracted from frozen tissues via QIAzol Lysis Reagent (#79306; Qiagen) was run
1024 through RNeasy spin columns following the RNeasy Lipid Tissue Mini Kit in an automated
1025 manner with the QIAcube Connect (#9002864; Qiagen). Integrity was confirmed using RIN
1026 values with a cut-off of 8. Libraries were prepared by the Next Gen Sequencing Core at CSHL
1027 using the Illumina TruSeq mRNA Stranded Sample prep kit (96 index High Throughput) and
1028 normalized using Kapa Biosystem's Library Quantification Kit. NextSeq High Output Paired-End
1029 150bp was run for sequencing.

1030 For the analysis, reads were aligned to the mouse genome version GRCm38.74 and read
1031 counts were obtained using "biomaRt" R package. "org.Mm.eg.db" R package was used for
1032 genome wide annotation. Read counts were normalized and tested for differential gene
1033 expression using the Bioconductor package "edgeR". Multiple testing correction was applied
1034 using the Benjamini-Hochberg procedure (FDR <0.05). "fgsea" and "dplyr" packages were used
1035 for GSEA in R. GSEA was performed by ranking all genes tested in RNA-Seq using -log10 (p-
1036 values) derived from differential expression analyses and testing against MSigDB Hallmark gene
1037 sets and Canonical pathways KEGG gene sets. Results were curated using a p-adj <0.05
1038 threshold.

1039 **Statistical analysis**

1040 Data were expressed as the mean \pm SEM unless otherwise stated and statistical
1041 significance was analyzed using GraphPad Prism 7.03 software. For survival analysis, data were
1042 shown as Kaplan Meier curves and the log-rank (Mantel–Cox) test was used to assess survival
1043 differences. When comparing more than 2 groups at the same time, one-way ANOVA with
1044 Tukey's correction for post-hoc testing was used. For statistical comparison of quantitative data
1045 at different times, unpaired two-tailed Student's t-tests were performed at each timepoint with
1046 the Holm-Sidak method correction for multiple comparisons. To analyze the main independent
1047 effect of diet and cancer and the interaction of both factors, two-way ANOVA tests were used,
1048 as well as Welch's t-test to compare two-samples with different variance.

1049 For global metabolic profiling of liver and tumor, a Principal Component Analysis (PCA)
1050 was performed. PCA is an unsupervised statistical method that reduces the dimension of the
1051 data by using an orthogonal transformation to convert a set of observations of possibly
1052 correlated variables into a set of variables called principal components. Each principal
1053 component is a linear combination of every metabolite and the principal components are
1054 uncorrelated. The number of principal components is equal to the number of observations This
1055 method permits visualization of how individual samples in a dataset differ from each other. The
1056 first principal component is computed by determining the coefficients of the metabolites that
1057 maximizes the variance of the linear combination. The second component finds the coefficients
1058 that maximize the variance with the condition that the second component is orthogonal to the
1059 first. The third component is orthogonal to the first two components and so on. The total
1060 variance is defined as the sum of the variances of the predicted values of each component (the
1061 variance is the square of the standard deviation), and for each component, the proportion of
1062 the total variance is computed. Samples with similar biochemical profiles cluster together
1063 whereas samples with distinct profiles segregate from one another.

1064 Differences in tumor growth were assessed by fitting a mixed effect model with
1065 coefficients for the intercept, slope and the difference in the slope between diets, and a
1066 random component for each individual mouse. Significance was assessed by testing whether
1067 the coefficient for the difference in the slope was significantly different from zero using a t-test.
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1081 SUPPLEMENTAL INFORMATION TITLES AND LEGENDS

1082 **Figure S1. Cachectic phenotype of C26 and KPC murine models.** (A) Graphical summary of the
1083 experimental protocol. (B-C) Weight trajectories of C26 tumor-bearing mice and littermate
1084 controls (C), and KPC tumor-bearing mice and PC controls (C) on KD or NF diets since they were
1085 enrolled into the study until they reached cachectic endpoint. (D-E) Organ weights of cachectic
1086 C26 tumor-bearing mice and littermates (n=10-15) (D), and cachectic KPC tumor-bearing mice
1087 and PC controls (n=9-10) (E) fed either KD or NF diets. (F-G) mRNA expression of the PPAR α
1088 target genes Acadm (F) and Hmgsc2 (G) in C26 tumor-bearing mice and littermate controls fed
1089 KD or NF (n=5-7). (H-I) Cumulative food intake of KD- or NF-fed C26-tumor bearing mice and
1090 littermates (n=5 LM, n=12 C26) (H), and KD- or NF-fed KPC tumor-bearing mice and PC controls
1091 (n=10) (I) during the last 4 days before endpoint.

1092 Data are expressed as the mean \pm SEM. One-way ANOVA with Tukey's correction for post hoc
1093 testing was used in (D-G). Two-way ANOVA statistical tests with Tukey's correction for post hoc
1094 comparisons were performed in (H-I). * p-value < 0.05, ** p-value < 0.01, **** p-value <
1095 0.0001.

1096 **Supplemental Table 1. Macronutrient composition and caloric profile of standard and**
1097 **ketogenic diets.**

1098 **Figure S2. Intratumoral accumulation of lipids and saturation of the GSH system causes**
1099 **ferroptotic cell death.** (A) Schematic representation of the GSH pathway for detoxification of
1100 LPPs. (B) PCA of tumors from C26 tumor-bearing mice fed NF or KD (n=7). (B) PCA of untargeted
1101 metabolomics in the tumors of C26 mice fed KD or NF. (C-F) Quantification of ophthalmate (C),
1102 carnosine (D), hypotaurine (E) and taurine (F) metabolites by UPLC-MS/MS in the tumor of C26
1103 mice fed KD or NF (n=7). (G) Quantification by UPLC-MS/MS of GSH/GSSG ratio in the tumor of
1104 C26 mice fed KD or NF, untreated or treated with NAC (n=5-7). (H) Oil-Red-O staining of tumors
1105 from C26 mice on KD or NF. (I) Western blot of tumor lysates from C26 mice fed KD or NF

1106 stained for Caspase-3 and BAX apoptotic markers (n=5). (J) Immunohistochemistry staining of
1107 tumors from C26 mice fed KD or NF with the proliferation marker Ki67. (K-N) Quantification by
1108 flow cytometry of neutrophils (K), T-cells (L), macrophages (M) and monocytes (N), in the tumor
1109 of C26 mice fed KD or NF (n=3-4).

1110 Data are expressed as the mean ± SEM. Statistical analysis in (B) is described in the Methods
1111 section/Chapter 4. Statistical differences in (C-F, K-N) were examined using an unpaired two-
1112 tailed Student's t-test with Welch's correction. One-way ANOVA with Tukey's correction for
1113 post hoc testing was used in (G). * p-value < 0.05, ** p-value < 0.01, **** p-value < 0.0001.
1114

1115 **Figure S3. Biochemical deficiency in the corticosterone synthesis pathway in the adrenal**
1116 **cortex of tumor-bearing mice fed ketogenic diet.** (A) Murine synthetic pathway of
1117 corticosterone in the cortex of the adrenal glands. (B) Corticosterone levels at baseline (prior to
1118 diet change), 4 days after the start of the experiment, and at endpoint (cachexia) in C26 tumor-
1119 bearing mice and littermate controls fed KD or NF (n=5 LM, n=10-14 C26). (C) GSEA pathway
1120 analysis of cholesterol homeostasis and steroid biosynthesis in tumor-bearing KD-fed KPC mice
1121 compared to NF-fed KPC (n=5). (D) Plasma concentration of the pro-inflammatory cytokine IL-6
1122 in C26 tumor-bearing mice and control littermates on NF or KD diets at endpoint (n=5 LM, n=9-
1123 10 C26).

1124 Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's correction for post hoc
1125 testing was used in (B, D). Statistical analysis in (C) is described in Methods. * p-value < 0.05, **
1126 p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

1127 **Figure S4. Extended metabolic profiling of cachectic C26 and KPC mice.** (A-B) Plasma urea
1128 levels in cachectic C26 tumor-bearing mice and littermate controls (n=5 LM, n=10-11 C26) (A),
1129 and cachectic KPC tumor-bearing mice and PC controls (n=5-8) (B) fed either KD or NF diets. (C)
1130 PCA of hepatic metabolomics in C26 tumor-bearing mice and control littermates fed with KD or
1131 NF (n=5-6).

1132 Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's correction for post hoc
1133 testing was used in (A-B). Statistical analysis in (C) is described in Methods. * p-value < 0.05, **
1134 p-value < 0.01, **** p-value < 0.0001.

1135

1136 **Figure S5. Extended data on the systemic effects of Dexamethasone treatment.** (A) Weight
1137 trajectories of C26 tumor-bearing mice treated with Dexamethasone and fed with either KD or
1138 NF. (B) Survival of littermate controls, and C26 tumor-bearing mice treated or untreated with
1139 Dexamethasone, fed with KD or NF (n=7 LM, n=17-18 C26, n=7 C26 + Dex). (C) Percentage of
1140 mice in each group that were sacrificed because of cachexia (OS) or tumor size (PFS) endpoints.
1141 (D) Quantification by UPLC-MS/MS of metabolites involved in fatty acid metabolism in the liver
1142 of C26 tumor-bearing mice on either KD or NF diets, untreated or treated with Dexamethasone
1143 (n=5). (E) Organ weights of C26 tumor-bearing mice untreated or treated with Dexamethasone
1144 after 4 days of treatment (n=10-12 C26, n=3 C26 + Dex). (F-G) Cumulative water intake (F) and
1145 total movement (G) during the last 4 days before endpoint in littermate controls and C26
1146 tumor-bearing mice, untreated or treated with Dexamethasone, fed KD or NF (n=7).

1147 Data are expressed as the mean ± SEM. Survival: OS + PFS. Kaplan–Meier curves in (B-C) were
1148 statistically analyzed by using the log-rank (Mantel–Cox) test. One-way ANOVA with Tukey's
1149 correction for post hoc testing was used in (E). Analysis in (D, F-G) is described in Methods. * p-
1150 value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

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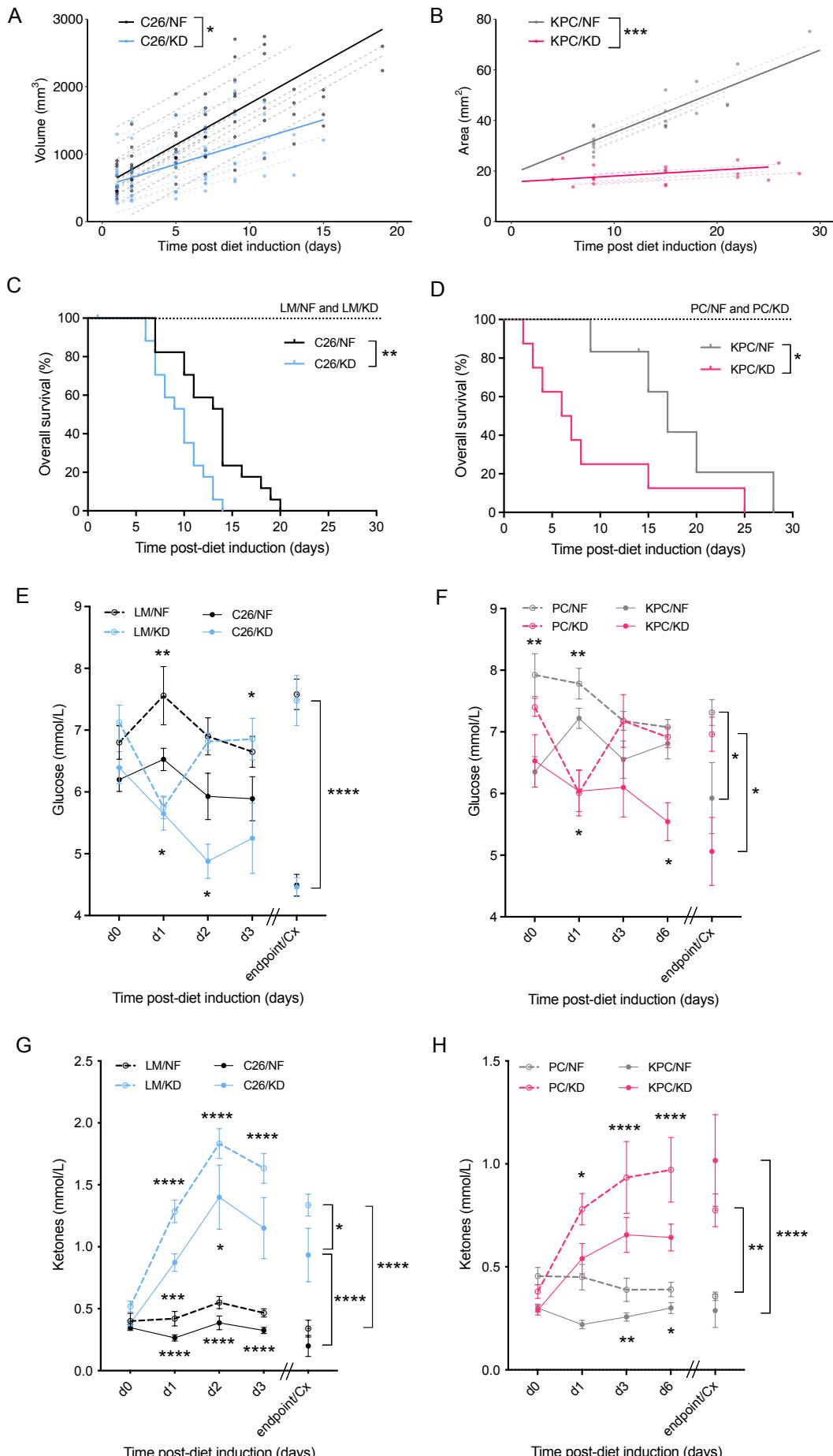


Figure 1

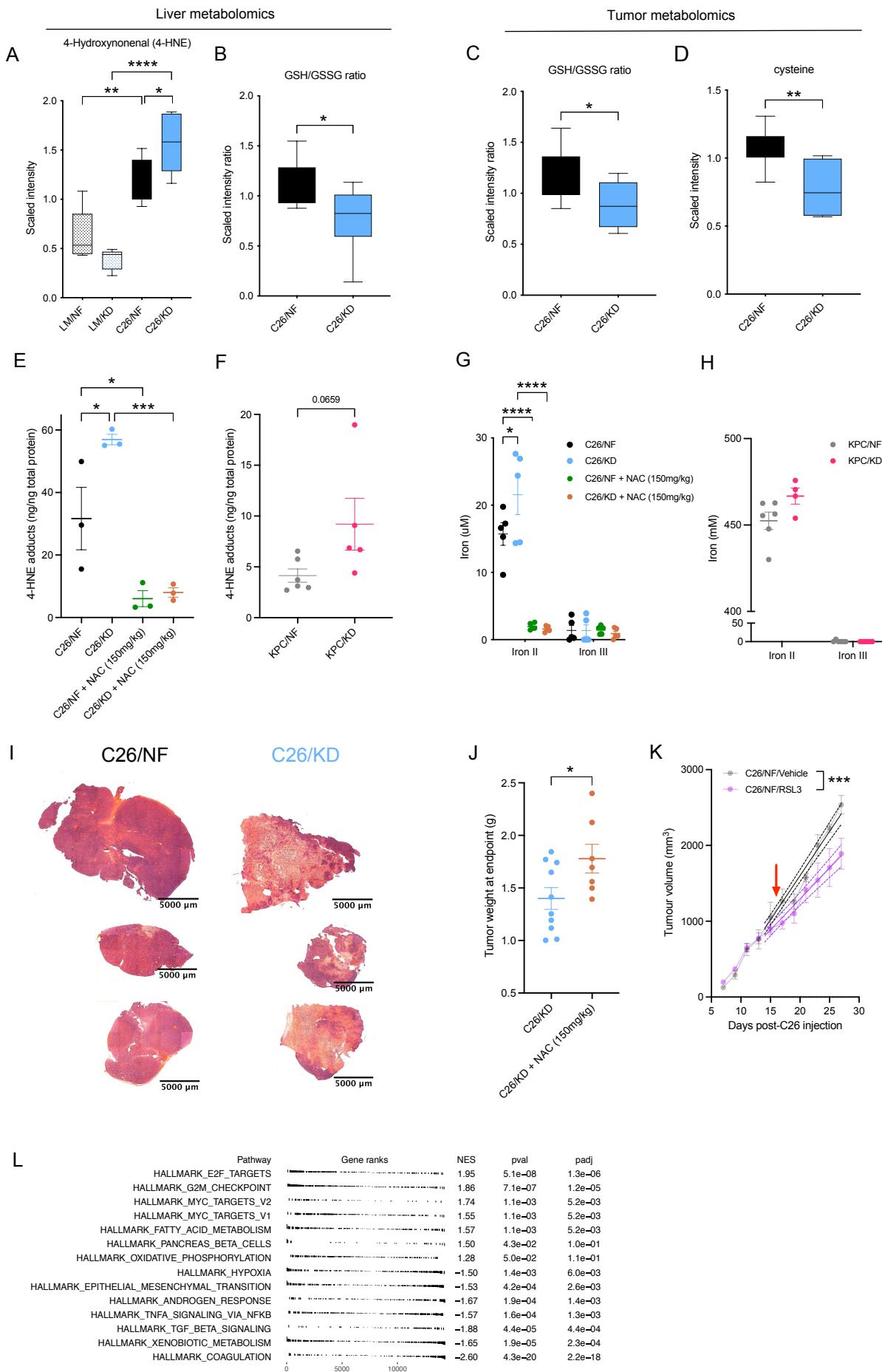


Figure 2

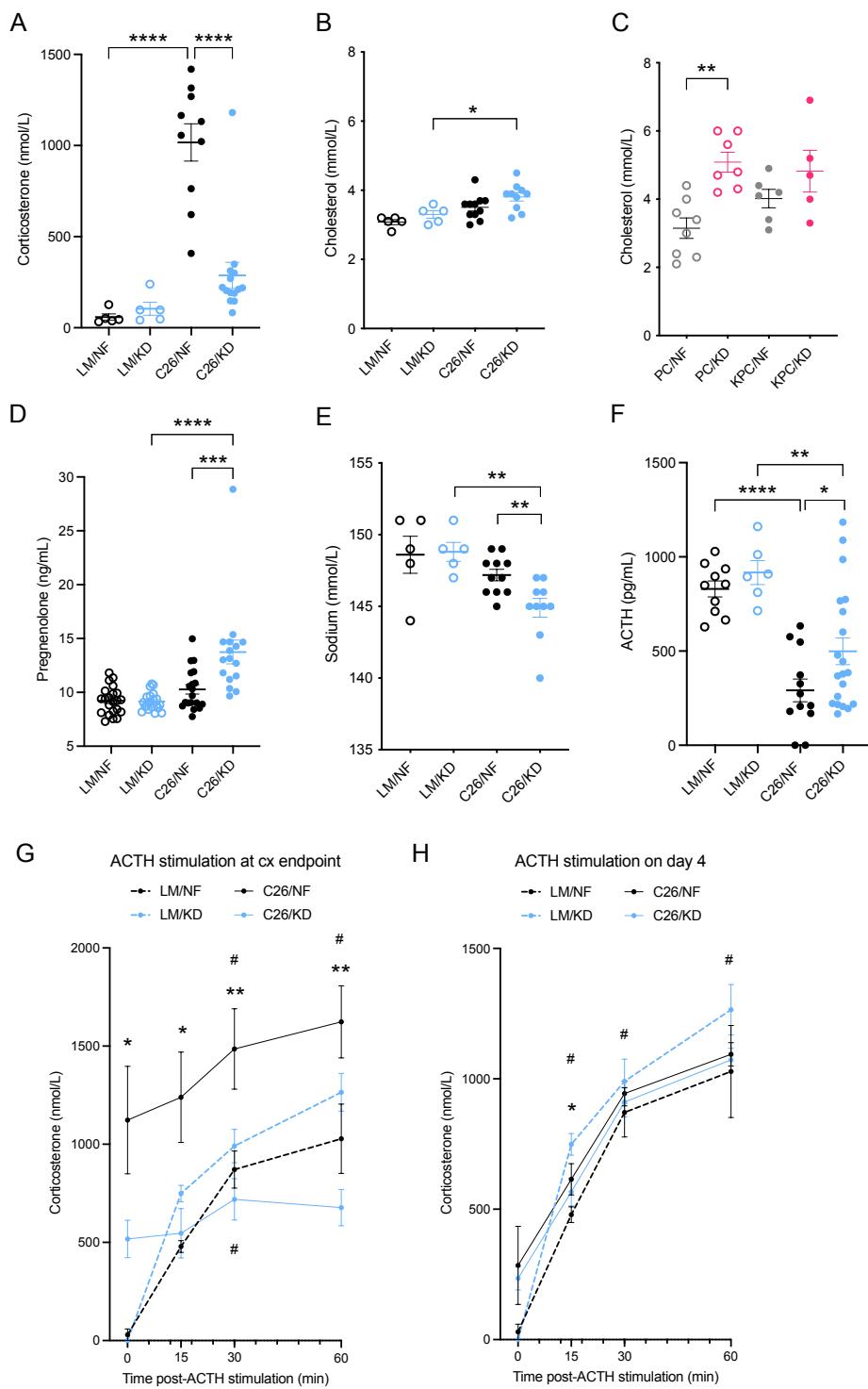


Figure 3

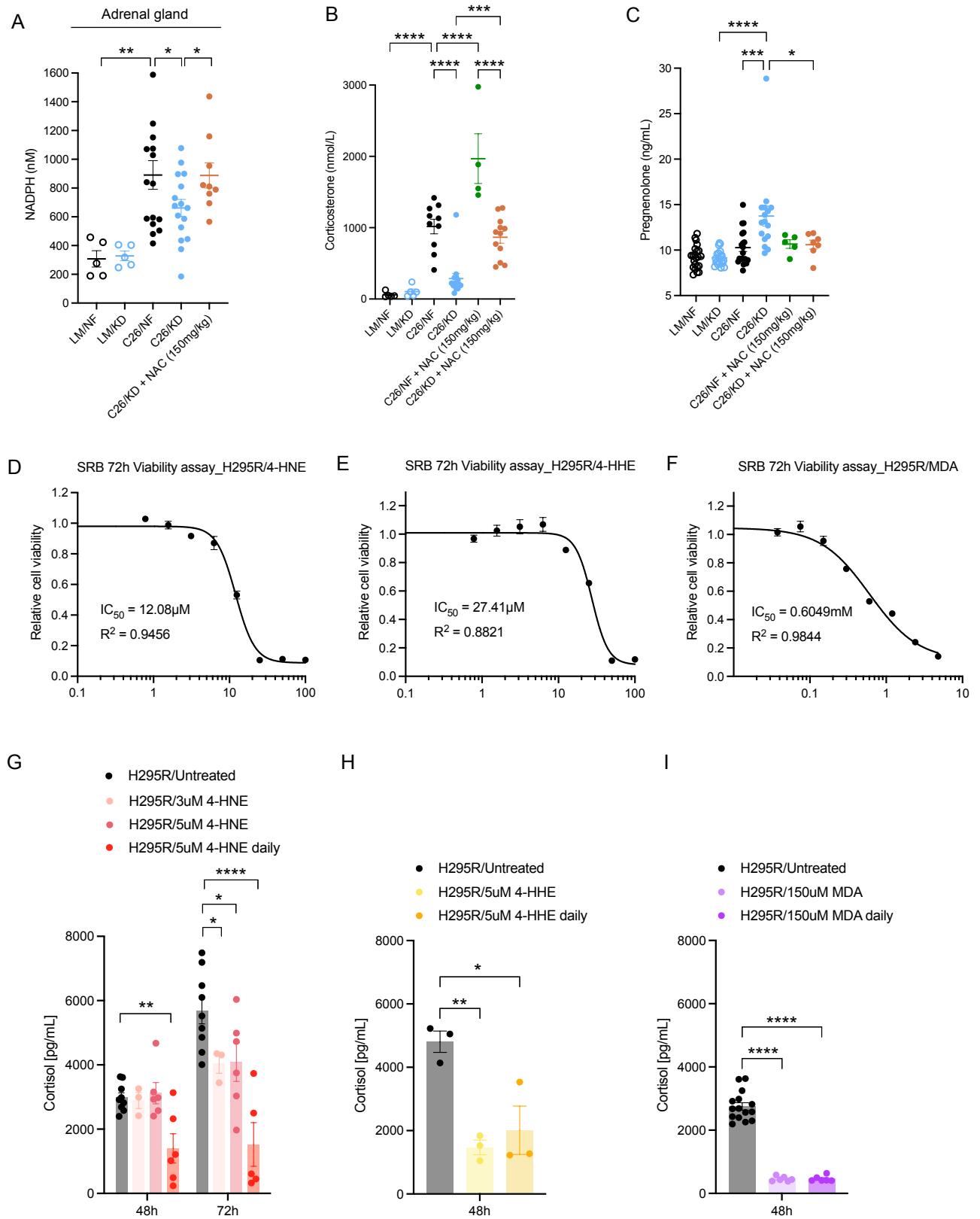


Figure 4

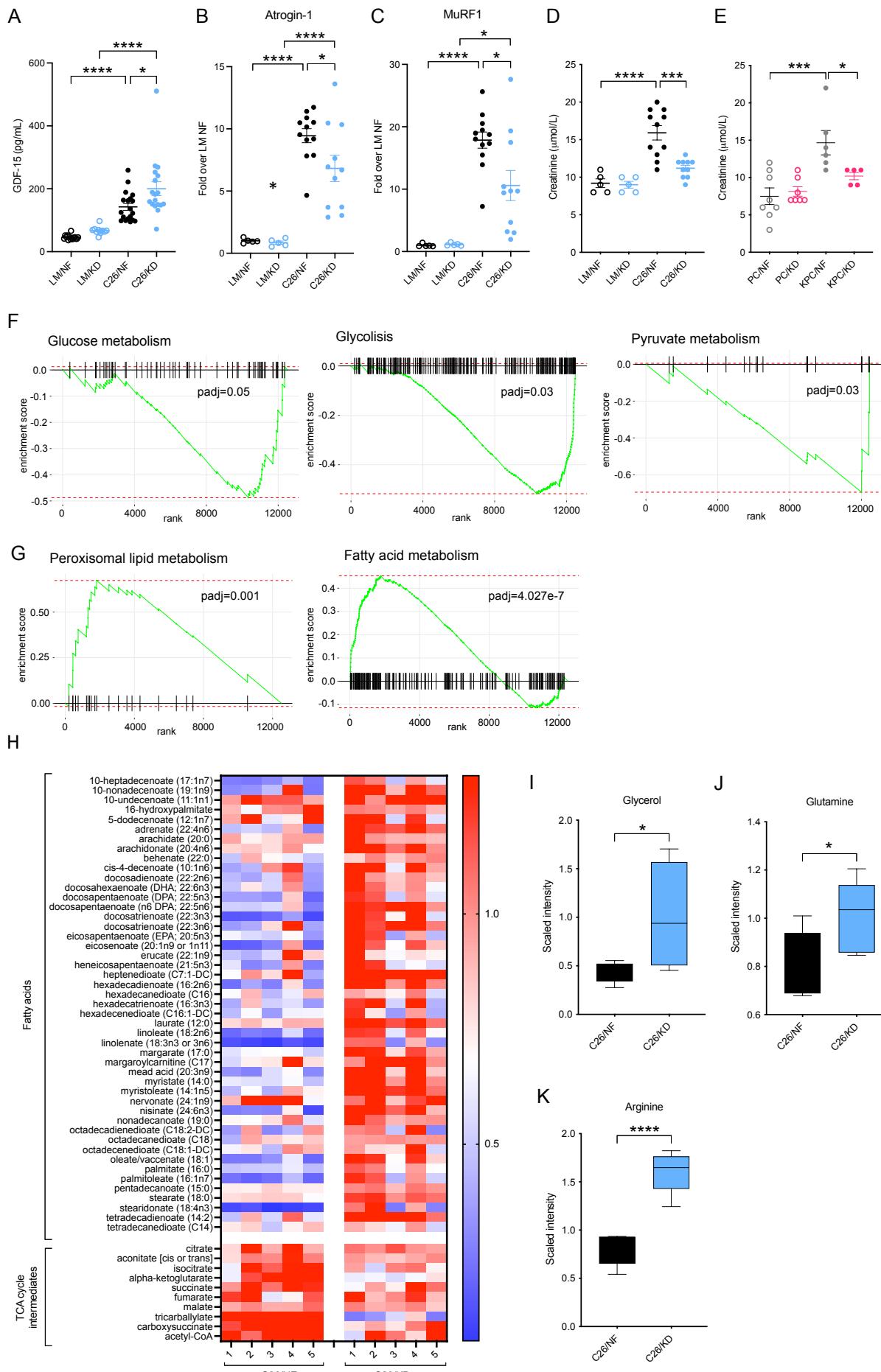
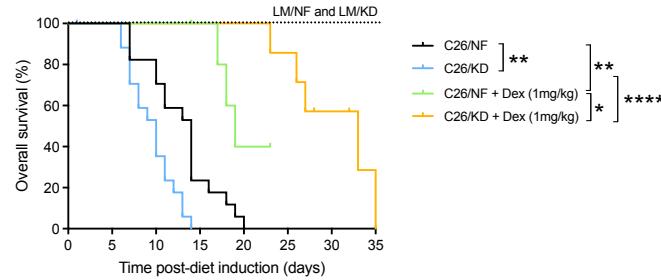
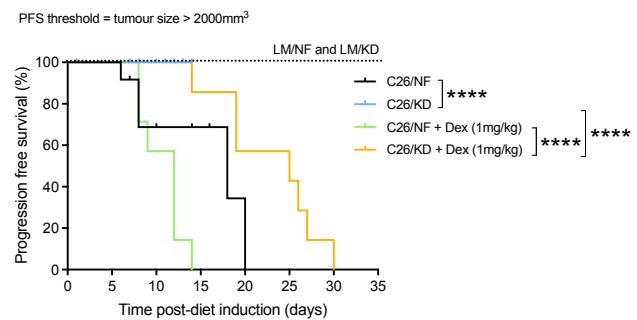


Figure 5

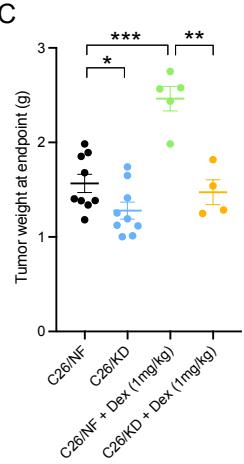
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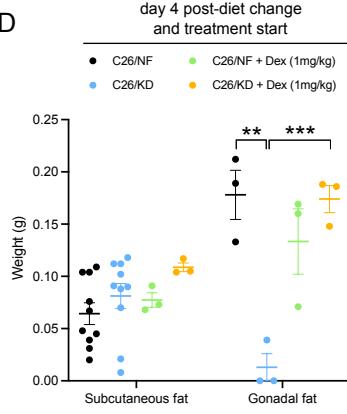
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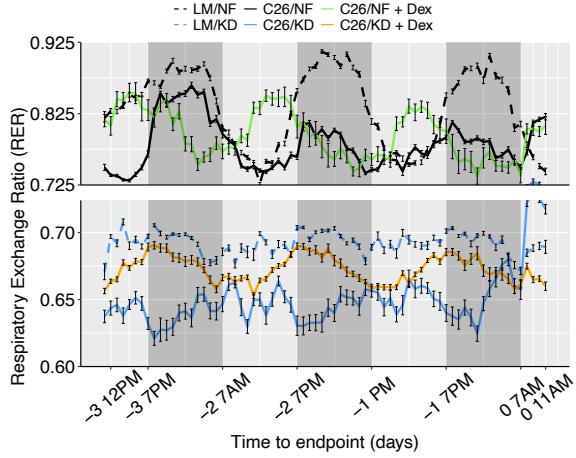
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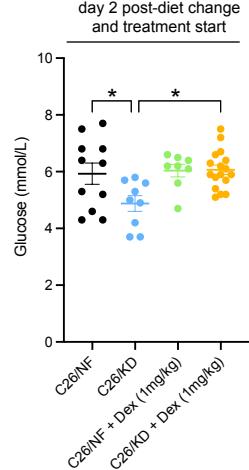
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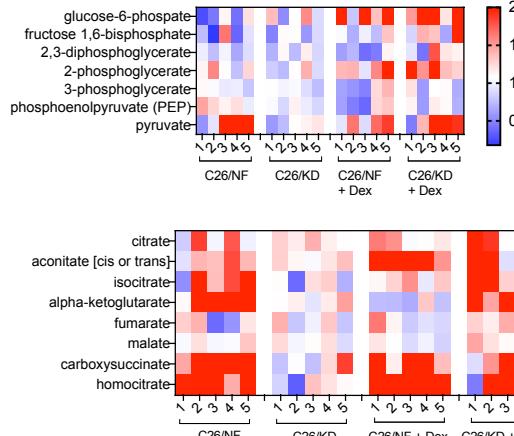
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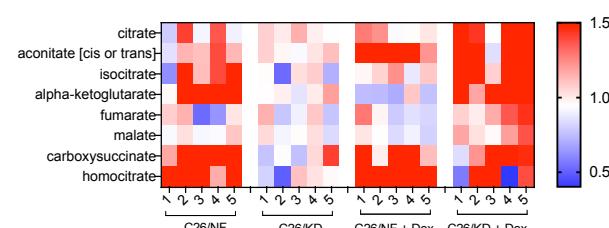
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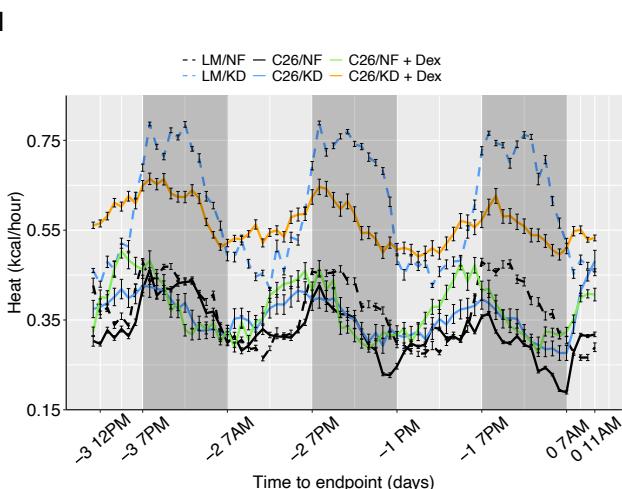
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H



I



J

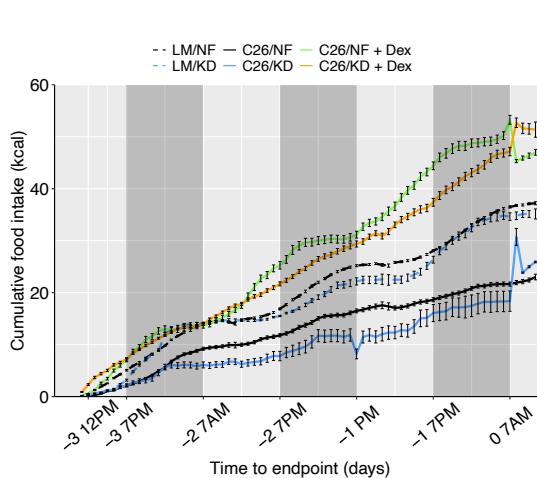
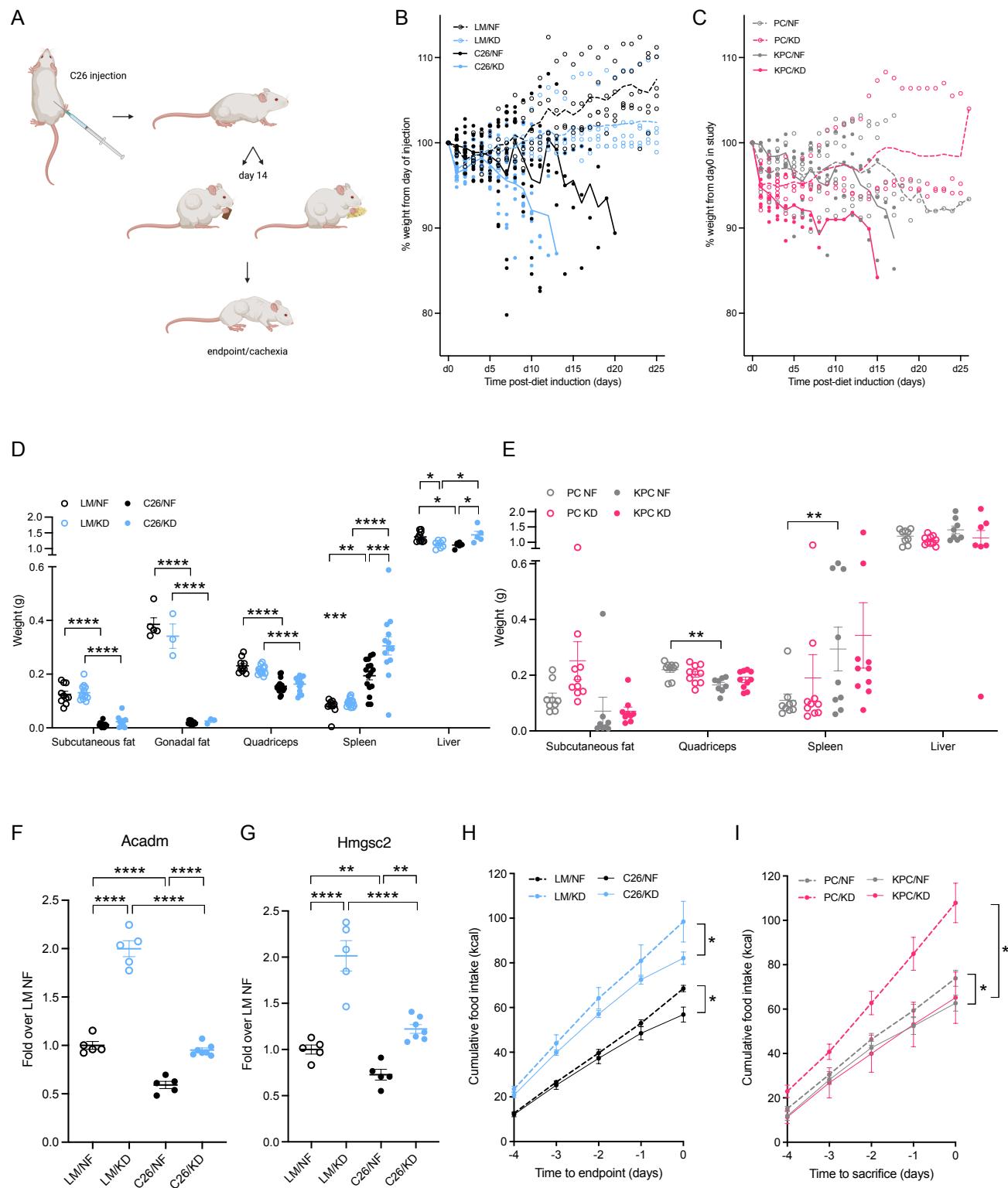
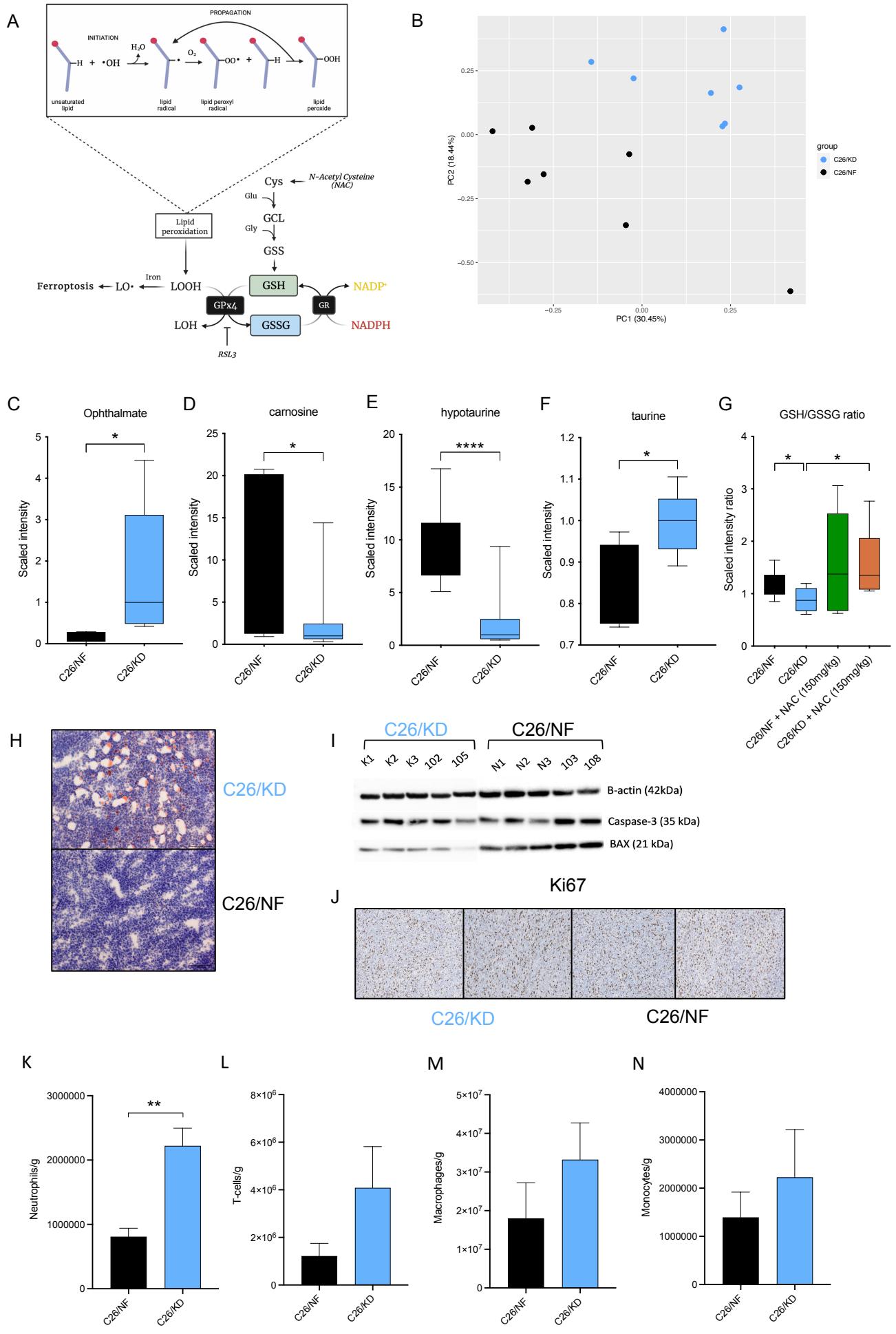


Figure 6

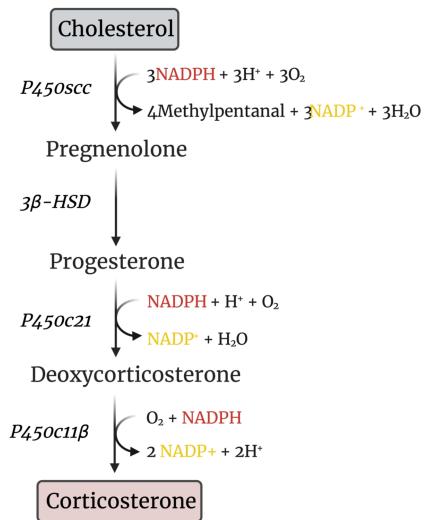


Supplementary Figure 1

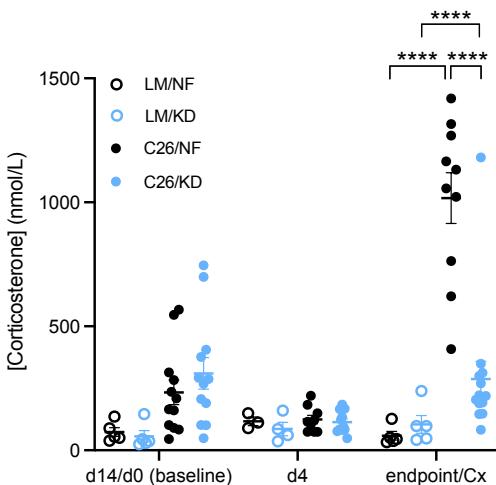


Supplementary Figure 2

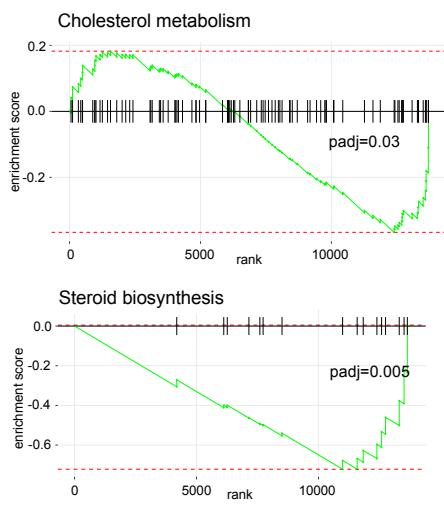
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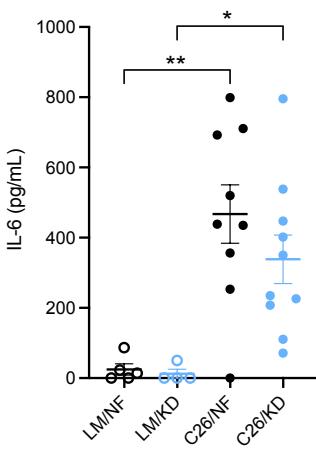
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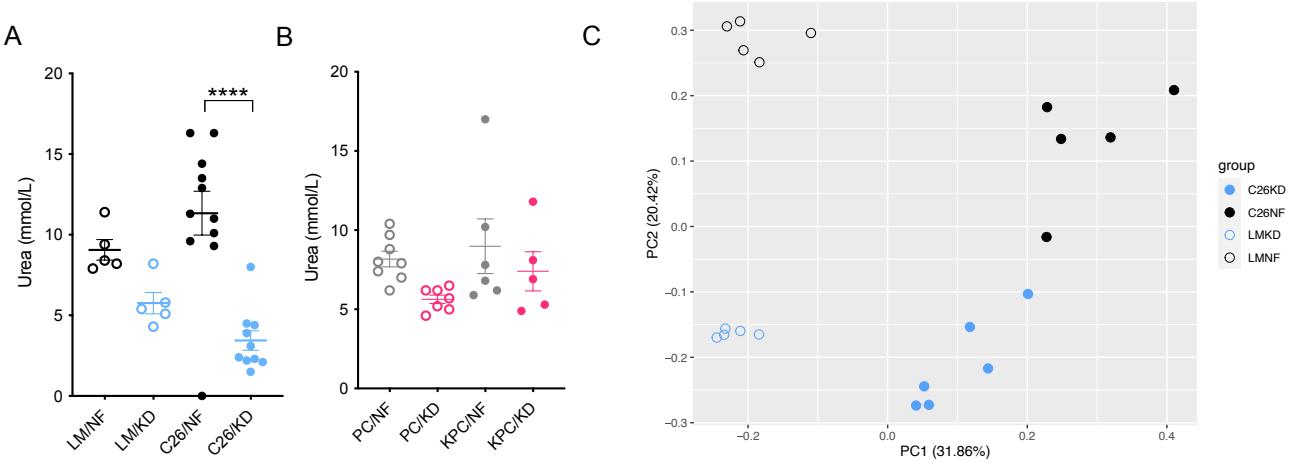
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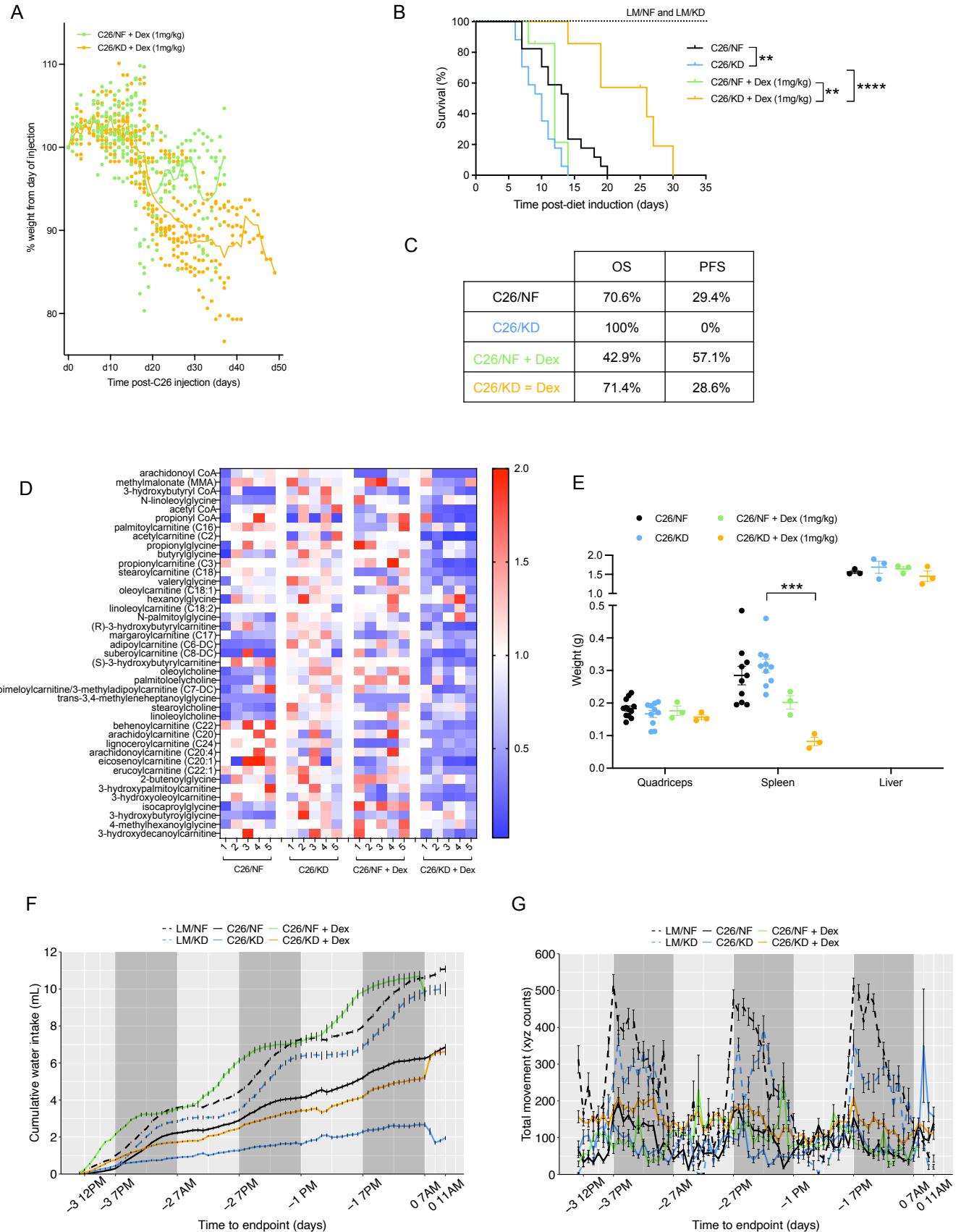
D



Supplementary Figure 3



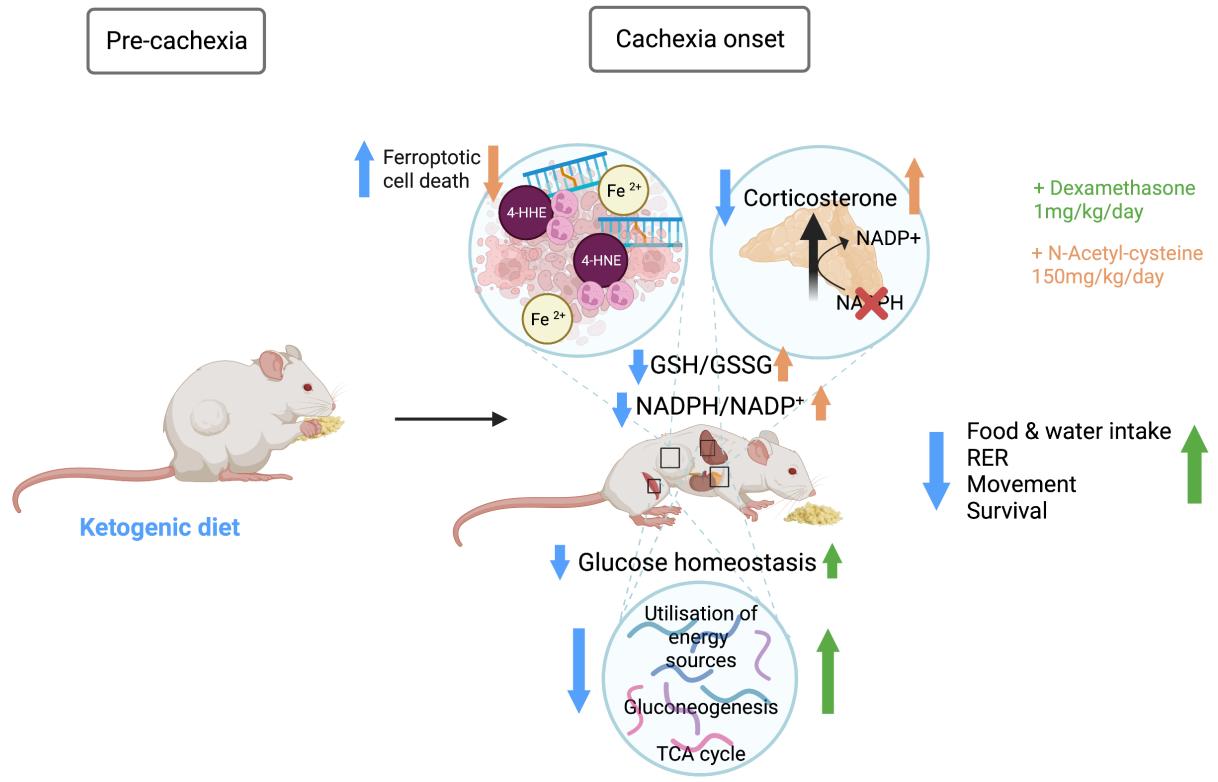
Supplementary Figure 4



Supplementary Figure 5

	Standard diet (PicoLab Rodent Diet 20)	Ketogenic diet (AIN-76A Modified)
Fat (%)	10.6	75.1
Protein (%)	20	8.6
Carbohydrates (%)	52.9	3.2
Fiber (%)	4.7	4.8
Ash (%)	6.1	3
Moisture (%)	<10	<10
Caloric profile (kcal/g)	4.07	7.24
Cystine (g/kg)	2.8	0.3
Methionine (g/kg)	7	2.2

Extended Data Table 1



Graphical abstract