

1 **Title:**

2 FGF21 protects against hepatic lipotoxicity and macrophage activation to attenuate
3 fibrogenesis in nonalcoholic steatohepatitis

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39 **Abstract**

40 Analogues of the hepatokine FGF21 are in clinical development for type 2 diabetes and
41 nonalcoholic steatohepatitis (NASH) treatment. Although their glucose-lowering and insulin-
42 sensitizing effects have been largely unraveled, the mechanisms by which they alleviate
43 liver injury have only been scarcely addressed. Here, we aimed to unveil the mechanisms
44 underlying the protective effects of FGF21 on NASH using APOE*3-Leiden.CETP mice, a
45 well-established model for human-like metabolic diseases. Liver-specific FGF21
46 overexpression was achieved in mice, followed by administration of a high-fat high-
47 cholesterol diet for 23 weeks. FGF21 prevented hepatic lipotoxicity, accompanied by
48 activation of thermogenic tissues and attenuation of adipose tissue inflammation,
49 improvement of hyperglycemia and hypertriglyceridemia, and upregulation of hepatic
50 programs involved in fatty acid oxidation and cholesterol removal. Furthermore, FGF21
51 inhibited hepatic inflammation, as evidenced by reduced Kupffer cell (KC) activation,
52 diminished monocyte infiltration and lowered accumulation of monocyte-derived
53 macrophages. Moreover, FGF21 decreased lipid- and scar-associated macrophages, which
54 correlated with less hepatic fibrosis as demonstrated by reduced collagen accumulation.
55 Collectively, hepatic FGF21 overexpression limits hepatic lipotoxicity, inflammation and
56 fibrogenesis. Mechanistically, FGF21 blocks hepatic lipid influx and accumulation through
57 combined endocrine and autocrine signaling, respectively, which prevents KC activation
58 and lowers the presence of lipid- and scar-associated macrophages to inhibit fibrogenesis.

59

60 **Keywords:** fibroblast growth factor 21; steatohepatitis; lipid/scar-associated macrophages;
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69 **Introduction**

70 The liver is the nexus of many metabolic pathways, including those of glucose, fatty acids
71 (FAs) and cholesterol. In health, these metabolites are distributed to peripheral tissues
72 while preventing long-lasting accumulation in the liver. In a pathological state, however,
73 lipids may accrue in the liver, thereby impairing liver function and carving the path towards
74 the development of nonalcoholic fatty liver disease (NAFLD) (1). NAFLD is considered a
75 spectrum of liver diseases ranging from liver steatosis, characterized by lipid accumulation
76 in hepatocytes, to nonalcoholic steatohepatitis (NASH) with hepatic steatosis, lobular
77 inflammation, hepatocyte ballooning and varying degrees of fibrosis (2, 3). Patients
78 diagnosed with NASH are predisposed to developing cirrhosis and hepatocellular
79 carcinoma, among whom patients with severe liver fibrosis are at greatest risk of overall-
80 and liver-related mortality (4). Despite this, there are currently no approved pharmaceutical
81 therapeutics for NASH. Instead, lifestyle modifications remain the first-line treatment for
82 NASH, although this is rarely attainable in the long term, and liver transplantation is still the
83 sole intervention to treat the end-stage of NASH (2, 5). Thus, there is an unmet need for
84 therapeutic strategies that control the progression of NASH, in particular of liver fibrosis,
85 and reverse the underlying pathophysiology.

86

87 Current hypotheses suggest that adipose tissue dysfunction and lipid spillover leads to
88 hepatic lipotoxicity, and thereby the initiation of NASH (6, 7), which further progresses
89 through the inflammatory response triggered by hepatic lipotoxicity (7). This inflammatory
90 response and subsequent fibrogenesis are primarily initiated by liver macrophages (8).
91 Hepatic macrophages mainly consist of embryonically-derived macrophages, termed
92 resident Kupffer cells (ResKCs), and monocyte-derived macrophages (MoDMacs) that are
93 recruited from the circulation (9). In the steady state, ResKCs serve as sentinels for liver
94 homeostasis. In NASH, liver injury caused by excess lipids and hepatocyte damage/death,

95 triggers ResKC activation, leading to pro-inflammatory cytokine and chemokine release (10).
96 This fosters the infiltration of newly-recruited monocytes into the liver, which gives rise to
97 various pro-inflammatory and pro-fibrotic macrophage subsets (8, 10). Interestingly, recent
98 preclinical and clinical studies have reported that modulation of ResKC activation, monocyte
99 recruitment or macrophage differentiation, to some extent, can attenuate NASH (8, 11). In
100 light of these findings, FGF21, a hepatokine with both lipid-lowering and anti-inflammatory
101 properties (12, 13), has been brought to the foreground as a promising potential therapeutic
102 to treat NASH.

103

104 The specificity of FGF21 action for various metabolic tissues is determined by the FGF
105 receptor (FGFR) which forms a heterodimer with the transmembrane co-receptor β -Klotho
106 (KLB) (14, 15). While the FGFR is ubiquitously expressed, KLB is primarily expressed in the
107 liver and adipose tissue (14, 15), therefore possibly limiting FGF21 action to these tissues.
108 Physiologically, FGF21 is considered a stress-induced hormone whose levels rise in
109 metabolically compromised states, such as obesity (16) and NASH (17). The increased
110 FGF21 in these pathologies is likely induced by an accumulation of lipids in the liver (18).
111 As such, plasma FGF21 also positively correlates with the severity of steatohepatitis and
112 fibrosis in patients with NASH (17). Induction of FGF21 is thought to mediate a
113 compensatory response to limit metabolic dysregulation (19), although this level is not
114 sufficient. Interestingly, two phase 2a clinical trials reported that pharmacological FGF21
115 treatment improves liver steatosis in NASH patients (20, 21). While an *in vivo* study testing
116 the therapeutic potency of FGF21 in choline-deficient and high-fat diet-induced NASH has
117 previously reported both anti-inflammatory and anti-fibrotic effects (22), detailed
118 mechanistic understanding is still lacking.

119

120 In the present study, we aimed to elucidate the mechanisms underlying FGF21-mediated
121 improvement of NASH, in particular of steatohepatitis and fibrogenesis. To this end, we
122 used APOE*3-Leiden.CETP mice, a well-established model for human cardiometabolic
123 diseases. These mice exhibit human-like lipoprotein metabolism, develop hyperlipidemia,
124 obesity and inflammation when fed a high-fat high-cholesterol diet (HFCD), and develop
125 fibrotic NASH closely resembling clinical features that accompany NASH in humans (23,
126 24). Moreover, these mice show human-like responses to both lipid-lowering and anti-
127 inflammatory therapeutics during the development of metabolic syndrome (25-28). Here,
128 we show that specific overexpression of FGF21 in the liver, resulting in increases
129 circulating FGF21 levels, activates hepatic signaling associated with FA oxidation and
130 cholesterol removal. In parallel, FGF21 activates thermogenic tissues and reduces
131 adipose tissue inflammation, thereby protecting against adipose tissue dysfunction,
132 hyperglycemia and hypertriglyceridemia. As a consequence, FGF21 largely limits lipid
133 accumulation in the liver and potently blocks hepatic KC activation and monocyte
134 recruitment, thereby preventing the accumulation of pro-inflammatory macrophages in the
135 liver. In addition, FGF21 reduced the number of pro-fibrotic macrophages in the injured
136 liver, potentially explaining why FGF21 counteracts all features of NASH, including hepatic
137 steatosis, inflammation and fibrogenesis.

138 **Results**

139 **Liver-specific FGF21 overexpression increases circulating FGF21 levels and protects**
140 **against HFCD-induced body fat mass gain**

141 We aimed to elucidate the underlying mechanisms of FGF21-mediated hepatoprotective
142 effects on NASH, by using APOE*3-Leiden.CETP mice fed with a HFCD, a model that
143 induces all stages of NASH in a human-like fashion and recapitulates the ultrastructural
144 changes observed in NASH patients (23, 24). Since the liver is the main contributor to
145 circulating FGF21 (14), we employed an adeno-associated virus vector 8 (AAV8) vector
146 expressing codon-optimized FGF21 to induce liver-specific FGF21 overexpression in
147 APOE*3-Leiden.CETP mice. Mice treated with either AAV8-FGF21 or AAV8-null as controls
148 were fed with a HFCD for 23 weeks (**Figure 1A**). We confirmed liver-specific FGF21
149 overexpression by a large increase in *Fgf21* expression in the liver but not in adipose tissue,
150 resulting in high circulating FGF21 levels that persisted throughout the study (**Figure 1B**).
151 HFCD progressively and profoundly increased body weight over the experimental period,
152 accompanied by increased white adipose tissue (WAT) and brown adipose tissue (BAT)
153 weights relative to those of low fat low cholesterol (LFCD)-fed mice (**Figure 1C,D**). In
154 favorable contrast, FGF21 reduced body weight in the first 3 weeks, after which body
155 weight stabilized and remained lower than that of LFCD- and HFCD-fed mice by the end of
156 the study (-18% and -35%, respectively; **Figure 1C**). Concomitantly, FGF21 decreased
157 weights of gonadal WAT (gWAT; -67%), subcutaneous WAT (sWAT; -55%), interscapular
158 BAT (iBAT; -41%) and subscapular BAT (-41%) to levels comparable to those observed in
159 LFCD-fed mice (**Figure 1D**). These findings thus highlight the potent effects of FGF21 on
160 preventing fat mass gain under NASH-inducing dietary conditions.

161

162 **FGF21 protects against HFCD-induced adipose tissue dysfunction**

163 The profound fat mass-lowering effects of liver-derived FGF21 prompted us to examine its
164 role in adipose tissue function. Since we and others have previously shown that FGF21
165 activates thermogenic adipose tissues (29, 30), we first performed histological analyses of
166 BAT and sWAT, the adipose tissue that is most prone to browning (31). We observed that
167 FGF21 prevented the HFCD-induced lipid overload in BAT (-66%) and increased
168 uncoupling protein-1 (UCP-1) expression compared with both the LFCD- and HFCD-fed
169 groups (+15% and +26%, respectively) (**Figure 2A**). In sWAT, FGF21 prevented HFCD-
170 induced adipocyte hypertrophy (-41%), and increased the UCP-1 content (+94%) (**Figure**
171 **2B**). Among the adipose tissue depots, gWAT is most prone to diet-induced inflammation,
172 and surgical removal of inflamed gWAT attenuates NASH in obese mice (32). Similar to
173 sWAT, FGF21 protected against HFCD-induced adipocyte enlargement (-52%) in gWAT
174 and in addition fully prevented the formation of crown-like structures (CLSs; -93%) (**Figure**
175 **2C**). In agreement with these findings, FGF21 suppressed the HFCD-induced expression of
176 adhesion G protein-coupled receptor E1 (*Adgre1*; -56%), encoding the macrophage surface
177 marker F4/80, in addition to decreased expression of the pro-inflammatory mediators tumor
178 necrosis factor α (*Tnfa*; -60%), interleukin-1 β (*Il1b*; -50%) and monocyte attractant
179 chemokine C-C motif ligand 2 (*Ccl2*; -60%) (**Figure 2D**). Besides, FGF21 tended to
180 upregulate *Klb* (+33%) and *Fgfr1* (+ 30%) expression compared to HFCD-fed mice (**Figure**
181 **2-figure supplement 1**). Moreover, consistent with the critical role of adiponectin in
182 mediating the therapeutic benefits of FGF21 in adipose tissue(22, 33), FGF21 increased
183 plasma adiponectin levels compared to both LFCD- and HFCD-fed mice (+93% and +133%,
184 respectively; **Figure 2E**). These combined findings thus indicate that FGF21 prevents
185 HFCD-induced adipose tissue dysfunction during NASH development.
186
187 **FGF21 alleviates HFCD-induced hyperglycemia and hypertriglyceridemia**

188 We next examined whether FGF21 confers its glucose and lipid lowering effects during
189 NASH development. While HFCD induced hyperglycemia as compared to LFCD, FGF21
190 normalized fasting plasma glucose compared to LFCD, which was accompanied by lower
191 glucose excursion after an intraperitoneal glucose tolerance test (**Figure 3A,B**). In addition,
192 FGF21 normalized the plasma insulin and Homeostatic Model Assessment for Insulin
193 Resistance index (**Figure 3C**), indicating that FGF21 restores insulin sensitivity to that
194 observed in LFCD-fed mice. FGF21 did not prevent the HFCD-induced increase of plasma
195 total cholesterol (TC) levels (**Figure 3-figure supplement 1A**), nor the distribution of
196 cholesterol over the various lipoproteins (**Figure 3-figure supplement 1B**). Nonetheless,
197 FGF21 strongly and consistently reduced fasting plasma triglyceride (TG) levels throughout
198 the experimental period compared with LFCD- and HFCD-fed mice (-67% and -58%; at
199 week 22), which was specific for very-low density lipoprotein (VLDL) and low density
200 lipoprotein (LDL) (**Figure 3D**). In addition, an oral lipid tolerance test revealed that FGF21
201 prevented HFCD-induced lipid intolerance (**Figure 3E**). Taken together, FGF21 prevents
202 the HFCD-induced increase in circulating glucose and reduces circulating TG levels beyond
203 those observed in LFCD-fed mice.

204

205 **FGF21 protects against HFCD-induced hepatic steatosis, inflammation, and
206 fibrogenesis**

207 Then, we investigated the effects of FGF21 on liver steatosis, inflammation and fibrosis.
208 FGF21 not only prevented HFCD-induced liver weight gain (-58%), but even reduced liver
209 weight to a level lower than that of LFCD-fed mice (-40%; **Figure 4A,F**). Moreover, FGF21
210 abolished the HFCD-induced increase in steatosis, lobular inflammation and hepatocellular
211 ballooning (**Figure 4B, Figure 4-figure supplement 1A,B**). Therefore, FGF21 completely
212 prevented the HFCD-induced large increase in the NAFLD activity score (-74%; **Figure
213 4C,F**). Furthermore, FGF21 prevented collagen accumulation in the liver as assessed by

214 Picosirius Red staining (-58%; **Figure 4D,F**). We then measured hepatic concentration of
215 hydroxyproline, a major constituent of collagen and thus a marker of extracellular matrix
216 accumulation. In line with the hepatic collagen content, HFCD feeding increased the hepatic
217 hydroxyproline content, which was prevented by FGF21 (-49%; **Figure 4E**). Taken together,
218 our data demonstrate that FGF21 protects against HFCD-induced hepatosteatosis,
219 steatohepatitis as well as fibrogenesis.

220

221 **FGF21 abolishes liver lipotoxicity, accompanied by activation of hepatic signaling
222 involved in FA oxidation and cholesterol removal**

223 In the context of NASH, pro-inflammatory responses and fibrogenesis occur when
224 hepatocytes are injured by lipotoxicity (7, 34). Indeed, 23 weeks of HFCD feeding promoted
225 aberrant accumulation of TG as well as TC in the liver (**Figure 5A**). In agreement with the
226 data presented in **Figure 4**, FGF21 abrogated the HFCD-induced increase in hepatic TG
227 levels (-62%) and tended to decrease hepatic TC levels (-22%), resulting in smaller lipid
228 droplets (**Figure 5A**). In addition to reduced lipid overflow from WAT, we reasoned that
229 FGF21 may also directly act on the liver to prevent HFCD-induced liver lipotoxicity. In
230 agreement, compared to both LFCD- and HFCD-fed mice, FGF21 profoundly upregulated
231 the expression of *Klb* (+150% and +223%), *Fgfr1* (+57% and +79%), *Fgfr2* (+97% and
232 +77%), and *Fgfr4* (+53% and +67%) (**Figure 5-figure supplement 1**). We next quantified
233 the hepatic expression of key genes involved in FA and cholesterol handling. FGF21 did not
234 attenuate the HFCD-induced increased expression of FA translocase cluster of
235 differentiation 36 (*Cd36*) (**Figure 5-supplement 2A**). In favorable contrast, compared to
236 both LFCD- and HFCD-fed mice, FGF21 did increase the expression of carnitine palmitoyl
237 transferase 1 α (*Cpt1a*, +66% and +53%), peroxisome proliferator-activated receptor α
238 (*Ppara*, +67% and +53%) and peroxisome proliferator-activated receptor γ coactivator 1 α
239 (*Pgc1a*; +188% and +225%), all of those genes being key players involved in FA oxidation

240 (Figure 5B). Moreover, compared to LFCD- and HFCD-fed mice, FGF21 increased the
241 expression of apolipoprotein B (*Apob*, +26% and +38%), which is involved in VLDL
242 secretion (Figure 5-figure supplement 2B). Furthermore, FGF21 upregulated the
243 expression of ATP-binding cassette transporter G member 5 (*Abcg5*; 7-fold and 2-fold),
244 crucial for biliary secretion of neutral sterols (Figure 5C), increased the expression of
245 cholesterol 7 α -hydroxylase (*Cyp7a1*; +94% and +109%), a key gene involved in the classic
246 bile acid synthesis pathway (Figure 5D), and restored the expression of sterol 27-
247 hydroxylase (+38%), involved in the alternative bile acid pathway (Figure 5D). Considering
248 that bile acid synthesis is a major pathway for hepatic cholesterol disposal (35), FGF21
249 likely regulates bile acid metabolism to prevent HFCD-induced cholesterol accumulation in
250 the liver. Collectively, our data indicate that FGF21 increases the hepatic expression of key
251 genes involved in β -oxidation and cholesterol removal, which together with reduced lipid
252 overload from WAT may explain FGF21-induced alleviation of liver lipotoxicity under NASH-
253 inducing dietary conditions.

254

255 FGF21 prevents activation of various KC subsets

256 Then, we performed an in-depth phenotyping of hepatic immune cells using spectral flow
257 cytometry. For this, we developed a panel that identifies most major immune cell subsets
258 (for gating strategy see Figure 6-figure supplement 1A). As compared to LFCD, HFCD
259 tended to reduce total CD45 $^{+}$ leukocytes, which were increased by FGF21 (Figure 6-figure
260 supplement 1B). Combining conventional gating and dimension-reduction analysis through
261 uniform manifold approximation and projection allowed to identify FGF21-induced changes
262 in cell subset abundance (Figure 6A). FGF21 prevented HFCD-induced loss of eosinophils,
263 neutrophils and B cells, and increased numbers of dendritic cells and T cells compared with
264 those observed in both LFCD- and HFCD-fed mice (Figure 6-figure supplement 1B).
265 More importantly, FGF21 increased the number of total KCs compared with that of both

266 LFCD- and HFCD-fed mice (+63% and +156; **Figure 6-figure supplement 1B**), attenuated
267 HFCD-induced monocyte recruitment (-18%), and tended to repress the HFCD-induced
268 increase in hepatic MoDMacs (-42%; **Figure 6-figure supplement 1B**).

269

270 During the development of NASH, MoDMacs can gradually seed in KC pool by acquiring
271 ResKCs identity and replacing the dying ResKCs (36). These recruited MoKCs can have
272 both detrimental and supportive roles, contributing to increase in pathology during fibrosis
273 onset, but hastening recovery when the damage-evoking agent is attenuated/removed (37).
274 In light of this, we assessed the abundance and phenotype of ResKCs and monocyte-
275 derived KCs (MoKCs). We observed that FGF21 completely abolished the HFCD-induced
276 reduction of the number of ResKCs (+319%) and potently protected against HFCD-induced
277 ResKC activation as shown by decreased proportion of CD11c⁺ ResKCs (-53%; **Figure 6B**).

278 FGF21 also completely abolished the HFCD-induced upregulation of CD36 in ResKCs, to
279 levels that are even lower than those in LFCD-fed mice (-88% vs. LFCD; -94% vs. HFCD;
280 **Figure 6B**). In addition, FGF21 increased the number of MoKCs compared with that of both

281 LFCD- and HFCD-fed mice (+92% and +123%), and prevented the HFCD-induced increase
282 in the abundance of CD11c⁺ MoKCs (-42%) (**Figure 6C**). Strikingly, compared to both
283 LFCD- and HFCD-fed mice, FGF21 downregulated CD9 (-32% and -49%) and CD36 (-98%
284 and -100%) in MoKCs (**Figure 6C**). Furthermore, FGF21 profoundly repressed HFCD-
285 induced upregulation of hepatic *Tnfa* (-37%), *Il1b* (-41%) and *Ccl2* (-54%) expression to
286 levels comparable to those in LFCD-fed mice (**Figure 6D**), which is in line with the
287 observation that FGF21 prevents KC activation. Given that CD36^{hi} ResKCs and CD36^{hi}/
288 CD9^{hi} MoKCs are involved in the formation of hepatic CLSs(10, 37-39), we next assessed
289 CLSs and observed that FGF21 completely prevented the HFCD-induced formation of
290 CLSs in the liver (-93%; **Figure 6D**). These data demonstrate that FGF21 inhibits the
291 activation of ResKCs and MoKCs and prevents the accumulation of CD36^{hi} ResKCs and

292 CD36^{hi}/ CD9^{hi} MoKCs under dietary conditions that result in NASH, which likely contribute
293 to the beneficial effects of FGF21 on hepatic inflammation and fibrosis.

294

295 **FGF21 protects against COL1A1 accumulation, as predicted by the reduction of**
296 **CD36^{hi} KCs and CD9^{hi} KCs**

297 To further evaluate whether FGF21-induced reductions of lipid-associated macrophages
298 (i.e., CD36^{hi} ResKCs and CD36^{hi} MoKCs) (38) and scar-associated macrophages (i.e.,
299 CD9^{hi} MoKCs) (40), are implicated in fibrogenesis, we performed multiple univariate
300 regression analyses. These revealed that both NAFLD activity and liver fibrosis were
301 associated with both CD36^{hi} ResKCs, CD36^{hi} MoKCs and CD9^{hi} MoKCs (**Figure 6-figure**
302 **supplement 2A-D**), indicating that FGF21 likely improves liver fibrosis by reducing these
303 lipid- and scar-associated macrophages. To further understand the underlying mechanisms
304 by which FGF21 prevents liver fibrosis, we measured hepatic expression of key genes
305 involved in fibrogenesis (**Figure 6D**). FGF21 tended to decrease the expression of
306 connective tissue growth factor (*Ctgf*, -27%), a major fibrogenic factor, and normalized the
307 HFCD-induced increased expression of its downstream target collagen type I α 1 (*Col1a1*; -
308 61%; **Figure 6D**). This finding was confirmed by immunohistochemistry, revealing that
309 FGF21 reduced hepatic COL1A1 accumulation (-46%; **Figure 6D**). Furthermore, univariate
310 regression analysis revealed that COL1A1 expression is predicted by CD36^{hi} ResKCs,
311 CD36^{hi} MoKCs and CD9^{hi} MoKCs (**Figure 6E, Figure 6-figure supplement 2E**). Taken
312 together, these data indicate that FGF21 reduces lipid- and scar-associated macrophages
313 to inhibit COL1A1 synthesis and prevent fibrogenesis.

314 **Discussion**

315 Several FGF21 analogues are currently being evaluated in clinical trials for the treatment of
316 NASH (20, 21). While the protective effect of pharmacological intervention with long-acting
317 FGF21 on human liver steatosis has been uncovered (20, 21, 41), mechanisms underlying
318 attenuated steatosis as well all the anti-inflammatory and anti-fibrotic effects of FGF21 on
319 NASH are still largely unexplored. Therefore, we set out to elucidate mechanisms by which
320 FGF21 beneficially modulates these various aspects of NASH in HFCD-fed APOE*3-
321 Leiden.CETP mice, a well-established model for diet-induced NASH (23, 24). Based on our
322 findings, we propose that FGF21 attenuates liver lipotoxicity via endocrine signaling to
323 adipose tissue to induce thermogenesis, thereby preventing adipose tissue dysfunction to
324 reduce lipid overflow to the liver, as well as autocrine signaling to the liver to increase FA
325 oxidation and cholesterol removal. In addition, FGF21 prevents KC activation, monocyte
326 recruitment and the formation of lipid- and scar-associated macrophages, thereby likely
327 inhibiting collagen accumulation and alleviating liver fibrogenesis.

328

329 Hepatic lipotoxicity is one of the major risk factors determining the progression of liver
330 steatosis into NASH, as shown in multiple clinical studies with obese patients (42-44). By
331 feeding APOE*3-Leiden.CETP mice a diet rich in fat and cholesterol, we mimicked a
332 situation in which a positive energy balance induces many aspects of the metabolic
333 syndrome, including insulin resistance, obesity with increased fat accumulation, and hepatic
334 lipotoxicity indicated by hepatomegaly with aberrant accumulation of TG as well as TC.
335 Hepatic lipotoxicity likely results from lipid overflow from insulin-resistant adipose tissue
336 towards the liver in combination with hepatic insulin resistance that prevents insulin-
337 stimulated outflow of lipids (45). Within this dietary context, we applied a single
338 administration of an AAV8 vector encoding codon-optimized FGF21, which resulted in liver-
339 specific FGF21 overexpression. Since the codon-optimized FGF21 mitigates the poor

340 pharmacokinetic properties of native FGF21, including its short plasma half-life (0.5-2 hours)
341 by reducing proteolytic degradation(45), an elevated level of circulating FGF21 was
342 reached throughout the dietary intervention period. By this strategy, we mimicked the
343 situation in which circulating FGF21 predominantly derives from the liver (46). Indeed,
344 circulating FGF21 correlates well with the hepatic expression of FGF21 (47). Interestingly,
345 hepatic expression of FGF21 fully prevented the diet-induced increase in liver weight, liver
346 lipids (i.e., TG and TC) and steatosis score.

347

348 These lipotoxicity-protective effects of FGF21 can partially be explained by endocrine
349 effects of liver-derived FGF21 on adipose tissue, which besides the liver has high
350 expression of β -Klotho, the co-receptor of the FGFR (14, 15). Indeed, FGF21 fully
351 prevented the HFCD-induced increase in weights of WAT and BAT, with decreased lipid
352 accumulation in these adipose tissue depots as well as induction of BAT activation and
353 WAT browning. These data imply that FGF21 greatly induces thermogenesis which highly
354 increases energy expenditure, consistent with the thermogenic responses observed for
355 recombinant FGF21 in mice fed with an obesogenic diet (29) or atherogenic diet (30).
356 Activation of thermogenic tissues by classical β -adrenergic receptor largely increases the
357 uptake of circulating lipoprotein-derived FAs by BAT and beige WAT (48), which we
358 recently also demonstrated for recombinant FGF21 (30). This can thus at least partly
359 explain the marked TG-lowering effect of FGF21 observed in the current study.
360 Thermogenic activation also increases the uptake and combustion of glucose, although the
361 glucose-lowering and insulin-sensitizing effects of FGF21 can also be explained by
362 attenuated WAT inflammation in combination with increased adiponectin expression as well
363 as improved liver insulin sensitivity (30, 33, 49).

364

365 Besides endocrine FGF21 signaling in adipose tissue, liver lipotoxicity is likely further
366 prevented by autocrine FGF21 signaling. Indeed, we showed that liver-specific FGF21
367 overexpression increased hepatic expression of genes involved in FA oxidation (*Cpt1a*,
368 *Ppara*, *Pgc1a*), biliary cholesterol secretion (*Abcg5*), bile acids synthesis (*Cyp7a1*) and
369 VLDL production (*Apob*). Of note, these observations are in line with previous reports
370 showing increased FA oxidation (50) and upregulated *Abcg5* (51), *Cyp7a1* (51, 52) and
371 *Apob* (30) in the liver upon FGF21 treatment. Altogether, the marked protective effects of
372 FGF21 on HFCD-induced hepatic lipotoxicity likely results from combined endocrine and
373 autocrine signaling, leading to reduced lipid influx from adipose tissue to the liver coupled to
374 the activation of hepatic FA oxidation and cholesterol elimination pathways. Our
375 observations may likely explain the recent clinical findings that treatment with FGF21
376 analogues in patients with NASH not only reduced hepatic steatosis (20, 21) but also
377 increased hepatic bile acid synthesis and further promoted cholesterol removal, lowering
378 the risk for further hepatic lipotoxicity (53).

379

380 While NASH is initiated by hepatic lipotoxicity, NASH progression is mainly driven by
381 impaired KC homeostasis and subsequent liver inflammation (54). Therefore, we
382 investigated in depth the inflammatory response in the liver through a combination of
383 immunohistochemistry, flow cytometry and gene expression analyses. HFCD feeding
384 induced an array of inflammatory effects, including increased lobular inflammation,
385 hepatocyte ballooning and NAFLD activity scores as well as increased inflammatory foci
386 and CLSs, accompanied by a reduction in ResKCs with a relative increase in CD11c⁺
387 ResKCs, and an increase in MoDMacs and CD11c⁺ MoKCs. These observations are likely
388 explained by lipotoxicity-related damage to ResKCs, and release of TNF α , IL-1 β and MCP-
389 1 (*Ccl2*), both activating various downstream pro-inflammatory mediators as well as
390 promoting monocyte recruitment to remodel the KC pool(36, 55) and further exacerbating

391 hepatic inflammation (10, 38, 54, 56, 57). Importantly, FGF21 prevented most of these
392 HFCD-induced inflammatory responses, as it normalized lobular inflammation, hepatocyte
393 ballooning and NAFLD activity scores and CLSs, and reduced pro-inflammatory activation
394 of various KC subsets.

395

396 Fibrosis has been identified as the most important predictor of prognosis in NAFLD patients,
397 and therefore a main target in experimental pharmacological approaches (58). HFCD
398 feeding during 23 weeks induced early signs of fibrosis, as evident from an increased
399 *Col1a1* expression and COL1A1 content, accompanied by an increased content of the
400 hydroxyproline. Importantly, FGF21 blocked liver fibrogenesis, and decreased the
401 hydroxyproline content. These alterations were accompanied with reductions in lipid-
402 associated macrophages (i.e., CD36^{hi} ResKCs/MoKCs) (38) and scar-associated
403 macrophages (i.e., CD9^{hi} MoKCs) (40). In fact, when analysing the mouse groups together,
404 CD36^{hi} ResKCs/MoKCs and CD9^{hi} MoKCs positively correlated with liver fibrosis as
405 reflected by hydroxyproline content and COL1A-positive area, suggesting that these lipid-
406 and scar-associated macrophages are involved in fibrogenesis in our model. Indeed, high
407 numbers of CD9^{hi} macrophages have been found in fibrotic regions of the liver (37, 39, 40,
408 55), and these cells are able to prime quiescent primary murine hepatic stellate cells to
409 upregulate the expression of fibrillar collagen through CTGF (40), thereby promoting and
410 exacerbating liver fibrosis. Therefore, we speculate that FGF21 protects against early liver
411 fibrosis likely through preventing the accumulation of CD36^{hi}/CD9^{hi} KCs, thereby inhibiting
412 activation of hepatic stellate cells to produce collagen.

413

414 In conclusion, hepatic overexpression of FGF21 in APOE*3-Leiden.CETP mice limits diet-
415 induced hepatic lipotoxicity, inflammation and fibrogenesis. Through a combination of
416 endocrine and autocrine signaling, FGF21 reduces hepatic lipid influx and accumulation,

417 respectively. This results in reduced macrophage activation and monocyte recruitment with
418 less presence of lipid- and scar-associated macrophages, limiting activation of hepatic
419 stellate cells to produce collagen (for graphic summary see **Figure 6F**). As such, our
420 studies provide a mechanistic explanation for the hepatoprotective effects of FGF21
421 analogues in recent clinical trials including reduction in steatosis (20, 21, 53) as well as the
422 fibrotic marker N-terminal type III collagen pro-peptide (20, 21), and further highlight the
423 potential of FGF21 for clinical implementation as a therapeutic in the treatment of advanced
424 NASH.

425 **Materials and Methods**

426 Please see the **Supporting Information** for a detailed description of all experimental
427 procedures.

428

429 **Animals and treatments**

430 Male APOE*3-Leiden.CETP mice (on a C57BL/6J background) were generated as
431 previously described (59). Mice at the age of 10-12 weeks were group-housed (2-4 mice
432 per cage) under standard conditions (22°C, 12/12-hour light/dark cycle) with *ad libitum*
433 access to water and a LFCD (Standard Rodent Diet 801203, Special Diets Services, United
434 Kingdom), unless indicated otherwise. Then, based on body weight and 4-hour (9.00-13.00)
435 fasted plasma glucose, TG and TC levels, these mice were randomized into three treatment
436 groups (n = 18 per group), after which they received either AAV8-FGF21, a liver-tropic
437 AAV8 capsid vector expressing FGF21 under the control of a liver specific apolipoprotein E
438 /antitrypsin promoter (HFCD+FGF21 group; 2×10¹⁰ genome copies per mouse), or with the
439 same genome copy number of AAV8-null (HFCD and LFCD groups) via a single
440 intravenous injection. After one week of recovery, mice in the HFCD+FGF21 and HFCD
441 groups were switched to a HFCD (60% fat and 1% cholesterol; C1090-60, Altromin,
442 Germany) and maintained on the diet for 23 weeks. An intraperitoneal glucose tolerance
443 test (n = 8 per group) and an oral lipid tolerance test (n = 10 per group) were performed at
444 week 16 and week 20, respectively. Flow cytometry (n = 5 per group) was conducted at
445 week 23.

446

447 **Statistics**

448 Comparisons among three groups were analyzed using one-way ANOVA followed by a
449 Tukey post-test, unless indicated otherwise. Data are presented as mean ± SEM, and a *P*
450 value of less than 0.05 was considered statistically significant. All statistical analyses were

451 performed with GraphPad Prism 9.01 for Windows (GraphPad Software Inc., California, CA,
452 USA).

453

454 **Study approval**

455 All animal experiments were carried out according to the Institute for Laboratory Animal
456 Research Guide for the Care and Use of Laboratory Animals, and were approved by the
457 National Committee for Animal Experiments (Protocol No. AVD1160020173305) and by the
458 Ethics Committee on Animal Care and Experimentation of the Leiden University Medical
459 Center (Protocol No. PE.18.034.041).

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469

470 **Conflict of interest:**

471 ACA, AP, SO, MU, IA, YI, KW and XRP are employees of AstraZeneca.

472

473 **Data availability:**

474

475 All data generated or analyzed during this study are included in the manuscript and
476 supporting file.

477

478 **Author contributions:**

479 CL designed the study, carried out the research, analyzed and interpreted the results, and
480 wrote and revised the manuscript. MS interpreted the results, reviewed and revised the
481 manuscript and obtained the funding. BS and EZ carried out the research and reviewed the
482 manuscript. JML, HJPZ and BG designed and advised the study, interpreted the results and
483 reviewed the manuscript. MET advised the study and reviewed the manuscript. ACA, SO
484 and KW advised the study, interpreted the results and reviewed the manuscript. AP
485 designed AAV8-FGF21 vectors and edited the manuscript. MU and IA analyzed and

486 interpreted the results and reviewed the manuscript. YI and X-RP provided AAV8-FGF21
487 vectors, advised the study, interpreted the results and reviewed the manuscript. MRB
488 advised the study and reviewed the manuscript. YW designed and advised the study,
489 interpreted the results, reviewed and revised the manuscript. PCNR designed and advised
490 the study, interpreted the results, edited, reviewed and revised the manuscript and obtained
491 the funding.

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626

627 **Figure legends:**

628 **Figure 1. Liver-specific FGF21 overexpression increases circulating FGF21 levels**
629 **and protects against HFCD-induced body fat mass gain. (A)** Experimental set up. **(B)** At
630 week 23, FGF21 mRNA expression in the liver and gWAT was quantified (n = 16-18).
631 Plasma FGF21 levels were measured before (at week -1; pooled samples, n = 6 per group)
632 and after (at week 4, pooled samples, n = 6 per group; week 23, n = 12-16 per group)
633 AAV8-FGF21 administration. **(C)** Body weight was monitored throughout the experimental
634 period (n = 17-18). **(D)** At week 23, brown adipose tissue (BAT) and white adipose tissue
635 (WAT) depots were isolated and weighed (n = 18). Data are shown as mean \pm SEM.
636 Differences were assessed using one-way ANOVA followed by a Tukey post-test. *P < 0.05;
637 **P < 0.01, ***P < 0.001, compared with the LFCD group. #P < 0.001, compared with the
638 HFCD group. AAV8, adeno-associated virus 8; FGF21, fibroblast growth factor 21; gWAT,
639 gonadal WAT; HFCD, high fat and high cholesterol diet; iBAT, interscapular BAT; LFCD,
640 low fat and low cholesterol diet; sBAT, subscapular BAT; sWAT, subcutaneous white
641 adipose tissue.

642

643 **Figure 2. FGF21 protects against HFCD-induced adipose tissue dysfunction. (A)** In
644 iBAT, the lipid content and expression of uncoupling protein-1 (UCP-1) were quantified after
645 H&E staining and UCP-1 immunostaining, respectively. **(B)** In sWAT, the adipocyte
646 enlargement was assessed by H&E staining, and the tissue browning was evaluated by
647 UCP-1 immunostaining. **(C)** In gWAT, the adipocyte hypertrophy was detected, and the
648 number of CLSs was assessed, and **(D)** mRNA expression of pro-inflammatory markers
649 was quantified. **(E)** Plasma adiponectin concentration in fasted blood plasma was
650 measured at week 22. **(A)-(D)**, n = 14-18 per group; **(E)**, n = 10 per group. Differences were
651 assessed using one-way ANOVA followed by a Tukey post-test. *P < 0.05, ***P < 0.001,

652 compared with the LFCD group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the
653 HFCD group. *Adgre1*, adhesion G protein-coupled receptor E1; *Tnfa*, tumor necrosis factor
654 α ; *Il1b*, interleukin-1 β ; *Ccl2*, chemokine C–C motif ligand 2.

655

656 **Figure 3. FGF21 alleviates HFCD-induced hyperglycemia and hypertriglyceridemia. (A)**
657 Fasting plasma glucose levels were measured during the experimental period. **(B)** At week
658 16, an intraperitoneal glucose tolerance test (IPGTT) was initiated. **(B)** The area under the
659 curve (AUC) of plasma glucose during the IPGTT and **(C)** plasma insulin concentration in
660 response to the IPGTT was determined at the indicated timepoints. **(C)** Homeostasis model
661 assessment of insulin resistance (HOMA-IR) was determined from fasting glucose and
662 insulin levels. **(D)** Fasting plasma TG levels were measured throughout the study. The
663 distribution of triglyceride over lipoproteins was determined (pooled samples; $n = 5$ per
664 group) from plasma of week 22. **(E)** At week 20, an oral lipid tolerance test (OLTT) was
665 initiated, and AUC of plasma TG during the OLTT was calculated. **(A and D)**, $n = 14$ -18 per
666 group; **(B-C)**, $n = 7$ -8 per group; **(E)**, $n = 6$ -9 per group. Data are shown as mean \pm SEM.
667 Differences were assessed using one-way ANOVA followed by a Tukey post-test. $^{\ast}P <$
668 0.05 , $^{\ast\ast}P < 0.01$, $^{\ast\ast\ast}P < 0.001$, compared with the LFCD group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P <$
669 0.001 , compared with the HFCD group.

670

671 **Figure 4. FGF21 protects against HFCD-induced hepatic steatosis, inflammation and**
672 **fibrosis. (A)** At week 23, liver weight was determined, and **(B)** scoring of histological
673 features of steatosis, lobular inflammation and ballooning as well as **(C)** NAFLD activity was
674 evaluated by H&E staining. **(D)** Liver fibrosis was assessed by Picosirius Red (PSR)
675 staining, and **(E)** hepatic hydroxyproline levels were determined. **(F)** Representative
676 macroscopic, H&E and PSR pictures are shown. Data are shown as mean \pm SEM ($n = 16$ -
677 18 per group). Differences were assessed using one-way ANOVA followed by a Tukey

678 post-test. ${}^*P < 0.05$; ${}^{**}P < 0.01$, ${}^{***}P < 0.001$, compared with the LFCD group. ${}^{##}P < 0.01$;
679 ${}^{###}P < 0.001$, compared with the HFCD group.

680

681 **Figure 5. FGF21 abolishes liver lipotoxicity, accompanied by activation of hepatic**
682 **signaling involved in FA oxidation and cholesterol removal. (A)** Triglyceride (TG), total
683 cholesterol (TC) and phospholipid (PL) levels were determined in the liver (n = 18 per
684 group), and representative Oil Red O (ORO) pictures are shown. **(B)** The relative mRNA
685 expression of genes involved in fatty acid oxidation and **(C and D)** cholesterol removal (n =
686 15-18 per group) were determined in the liver. Data are shown as mean \pm SEM. Differences
687 were assessed using one-way ANOVA followed by a Tukey post-test. ${}^{**}P < 0.01$, ${}^{***}P <$
688 0.001, compared with the LFCD group. ${}^{##}P < 0.001$, compared with the HFCD group.
689 *Abcg5*, ATP-binding cassette transporter G member 5; *Cpt1a*, carnitine palmitoyl
690 transferase 1 α ; *Cyp7a1*, cholesterol 7 α -hydroxylase; *Cyp8b1*, sterol 12 α -hydroxylase;
691 *Cyp27a1*, sterol 27-hydroxylase; *Pgc1a*, peroxisome proliferator-activated receptor gamma
692 coactivator 1 α ; *Ppara*, peroxisome proliferator-activated receptor α .

693

694 **Figure 6. FGF21 modulates hepatic macrophage pool and protects against COL1A1**
695 **accumulation, as predicted by the reduction of CD36^{hi} KCs and CD9^{hi} KCs. (A)** Uniform
696 manifold approximation and projection for dimension reduction (UMAP) of immune cell
697 subsets from livers after 23-week of intervention. **(B)** The number of resident KCs (ResKCs),
698 the proportion of CD11c $^+$ ResKCs, and the expression of CD36 and CD9 in ResKCs were
699 quantified. **(C)** The amount of monocyte-derived KCs (MoKCs) was assessed, the
700 percentage of CD11c $^+$ MoKCs was determined, the CD36 and CD9 expression levels in
701 MoKCs were quantified. **(D)** Hepatic inflammation was evaluated by pro-inflammatory gene
702 expression and the formation of CLSs within the liver. The mRNA expression of liver

703 fibrogenesis markers was quantified, and the protein expression of collagen type 1 α 1
704 (COL1A1) was determined. (E) The expression of CD36 in ResKCs, and the expression of
705 CD9 and CD36 in MoKCs were plotted against COL1A1-positive area in the liver. (F)
706 Mechanistic model. Data are shown as mean \pm SEM (A-B and E, n = 4-5 per group; D, n =
707 16-18 per group). Linear regression analyses were performed. Differences were assessed
708 using one-way ANOVA followed by a Fisher's LSD test. *P < 0.05, **P < 0.01, ***P < 0.001,
709 compared with the LFCD group. $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, compared with the
710 HFCD group. *Acta2*, actin α 2; *Ctgf*, connective tissue growth factor; FA, fatty acid; *Tgfb1*,
711 transforming growth factor- β .

712

713 **Figure supplements:**

714 **Figure 2-figure supplement 1. Liver-specific FGF21 overexpression tends to
715 upregulate mRNA expression of FGF21 receptor 1 (FGFR1) and co-receptor β -Klotho
716 (KLB) in white adipose tissue (WAT).** The mRNA expression of KLB and FGFR1 in
717 gonadal WAT (gWAT). Data are shown as mean \pm SEM (n = 16-18 per group). Differences
718 were assessed using one-way ANOVA followed by a Tukey post-test.

719

720 **Figure 3-figure supplement 1. HFCD increases fasting cholesterol levels. (A)** Fasting
721 plasma total cholesterol (TC) levels were measured over a 23-week intervention period (n =
722 14-18 per group), and (B) the distribution of the cholesterol over circulating lipoproteins was
723 assessed at week 22 (pooled samples; n = 18 per group). Data are shown as mean \pm SEM.
724 Differences were assessed using one-way ANOVA followed by a Tukey post-test. ***P <
725 0.001, compared with the LFCD group. VLDL, very low-density lipoprotein; LDL, low-density
726 lipoprotein; HDL, high-density lipoprotein.

727

728 **Figure 4-figure supplement 1. FGF21 abolishes HFCD-induced increase of hepatic**
729 **lipid-positive area and the number of inflammatory foci.** At week 23, **(A)** hepatic lipid
730 droplet content and **(B)** inflammatory foci numbers were assessed by H&E staining. Data
731 are shown as mean \pm SEM (n = 18 per group). Differences were assessed using one-way
732 ANOVA followed by a Tukey post-test. **P < 0.01, ***P < 0.001, compared with the LFCD
733 group. #P < 0.01 ###P < 0.001, compared with the HFCD group.

734

735 **Figure 5-figure supplement 1. Liver-specific FGF21 overexpression upregulates**
736 **hepatic mRNA expression of FGF21 receptors (FGFRs) and co-receptor β -Klotho**
737 **(KLB).** The mRNA levels of KLB and FGFRs in the liver. Data are shown as mean \pm SEM
738 (n = 14-18 per group). Differences were assessed using one-way ANOVA followed by a
739 Tukey post-test. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the LFCD group. ##P <
740 0.01, ###P < 0.001, compared with the HFCD group.

741

742 **Figure 5-figure supplement 2. FGF21 increases apolipoprotein B mRNA (Apob)**
743 **expression in the liver.** At end of the study, hepatic expression of genes involved in **(A)**
744 fatty acid uptake and **(B)** VLDL production was quantified (n = 15-18 per group). Data are
745 shown as mean \pm SEM. Differences were assessed using one-way ANOVA followed by a
746 Tukey post-test. ***P < 0.001, compared with the LFCD group. ###P < 0.001, compared with
747 the HFCD group. *Apob*, apolipoprotein B; *Cd36*, cluster of differentiation 36; *Mttp*,
748 microsomal triglyceride transfer protein.

749

750 **Figure 6-figure supplement 1. FGF21 modulates the hepatic immune cell pool. (A)**
751 Flow cytometry gating strategy. **(B)** After 23 weeks of treatment, CD45⁺ cells were isolated
752 from the liver, and the number of CD45⁺ cells, eosinophils, neutrophils, B cells, dendritic
753 cells (DCs), T cells, natural killer (NK) cells, total Kupffer cells (KCs), Ly6C^{hi} monocytes and

754 monocyte-derived macrophages (MoDMacs) was assessed. Data are shown as mean ±
755 SEM (n = 4-5 per group). Differences were assessed using one-way ANOVA followed by a
756 Fisher's LSD test. *P < 0.05, **P < 0.01, compared with the LFCD group. #P < 0.05, ##P <
757 0.01, compared with the HFCD group.

758

759 **Figure 6-figure supplement 2. CD36^{hi} ResKCs as well as CD36^{hi}/CD9^{hi} MoKCs**
760 **positively correlate with NAFLD activity score and liver fibrosis.** NAFLD activity scores
761 and liver hydroxyproline levels were plotted against the expression of (A) CD9 and (B)
762 CD36 in ResKCs as well as (C) CD9 and (D) CD36 in MoKCs. (E) Hepatic expression of
763 collagen type 1α 1 (COL1A1) was plotted against the expression of CD9 in ResKCs. Linear
764 regression analyses were performed. Data are represented as mean ± SEM (n = 5 per
765 group).

766

767 **List of Supplementary Files:**

768 **Supplementary File 1:** Supporting Materials and Methods.

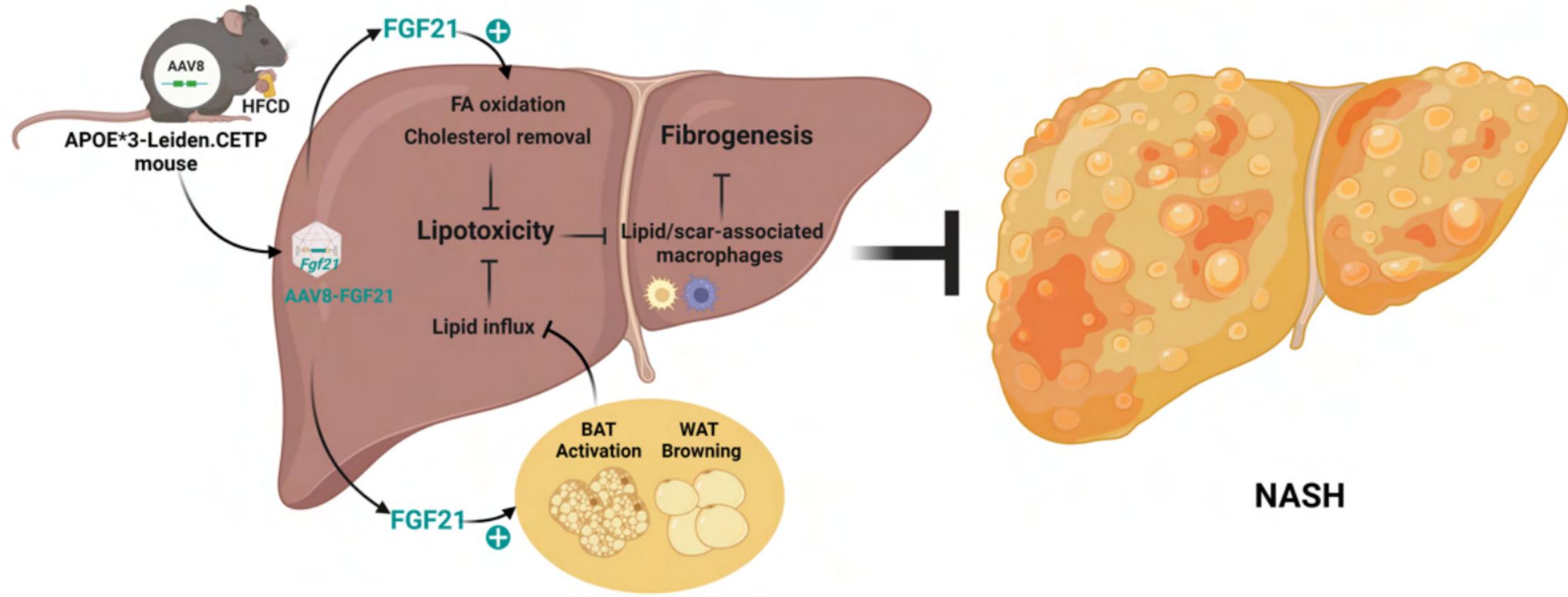


Figure 1

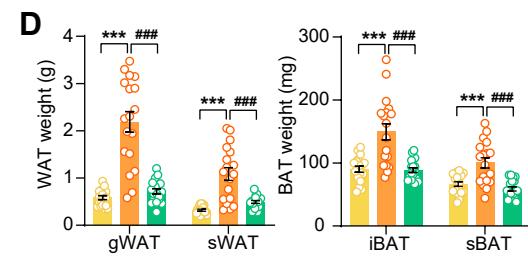
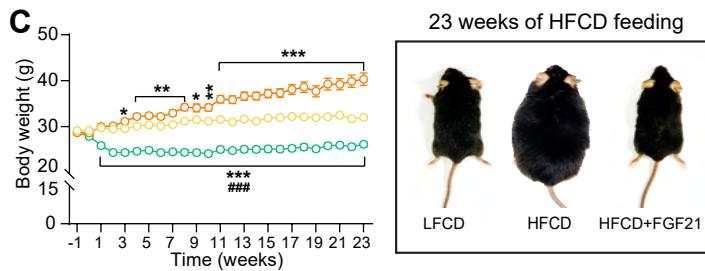
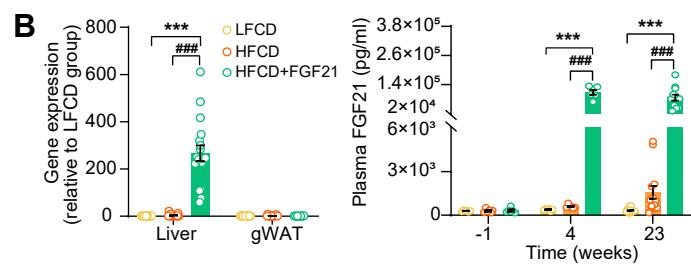
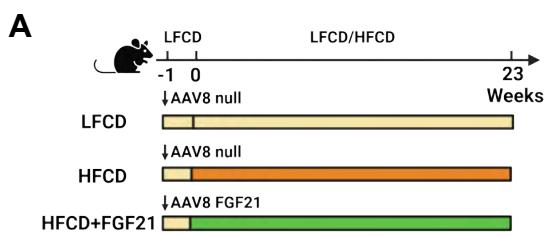


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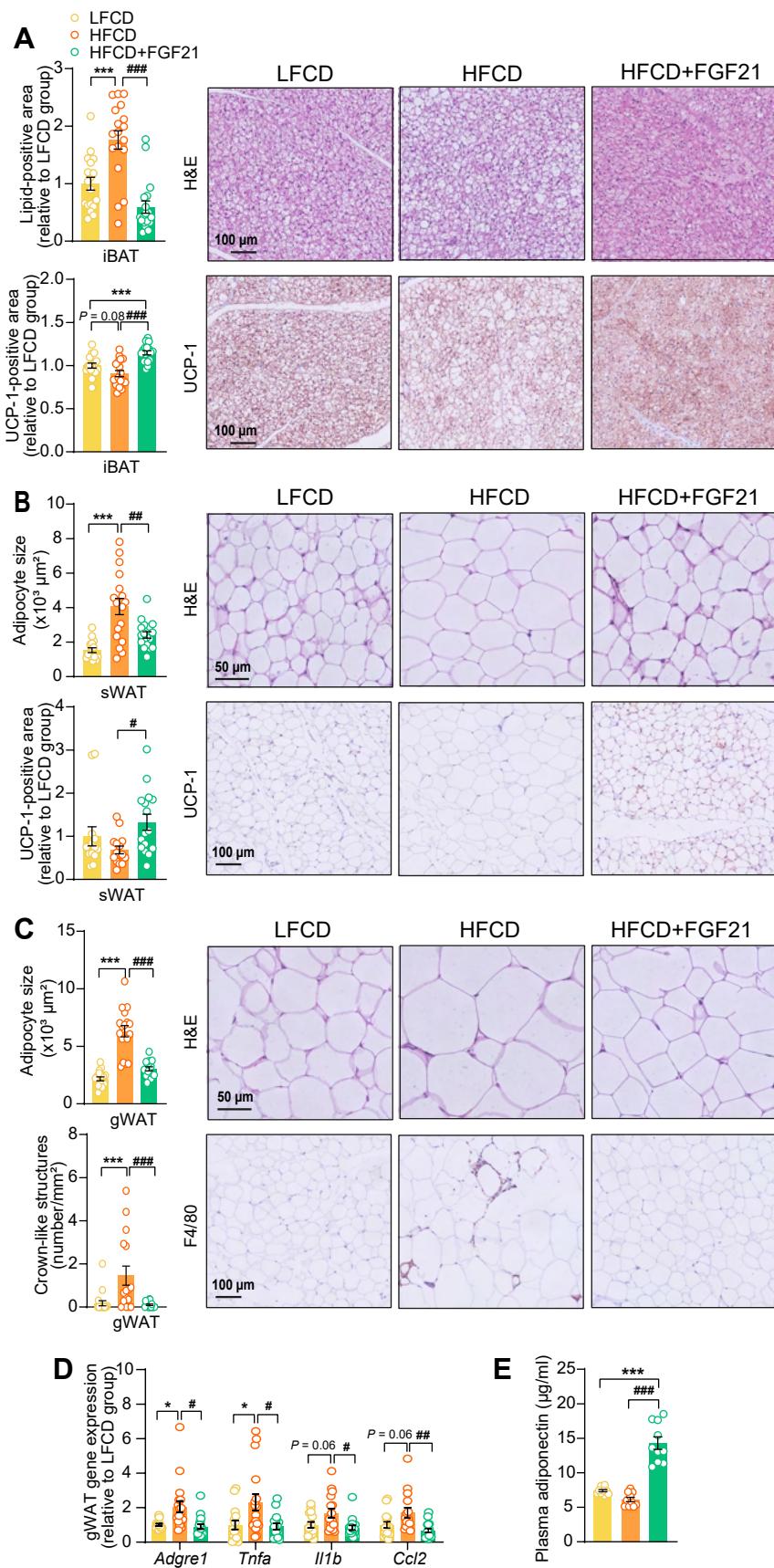


Figure 3

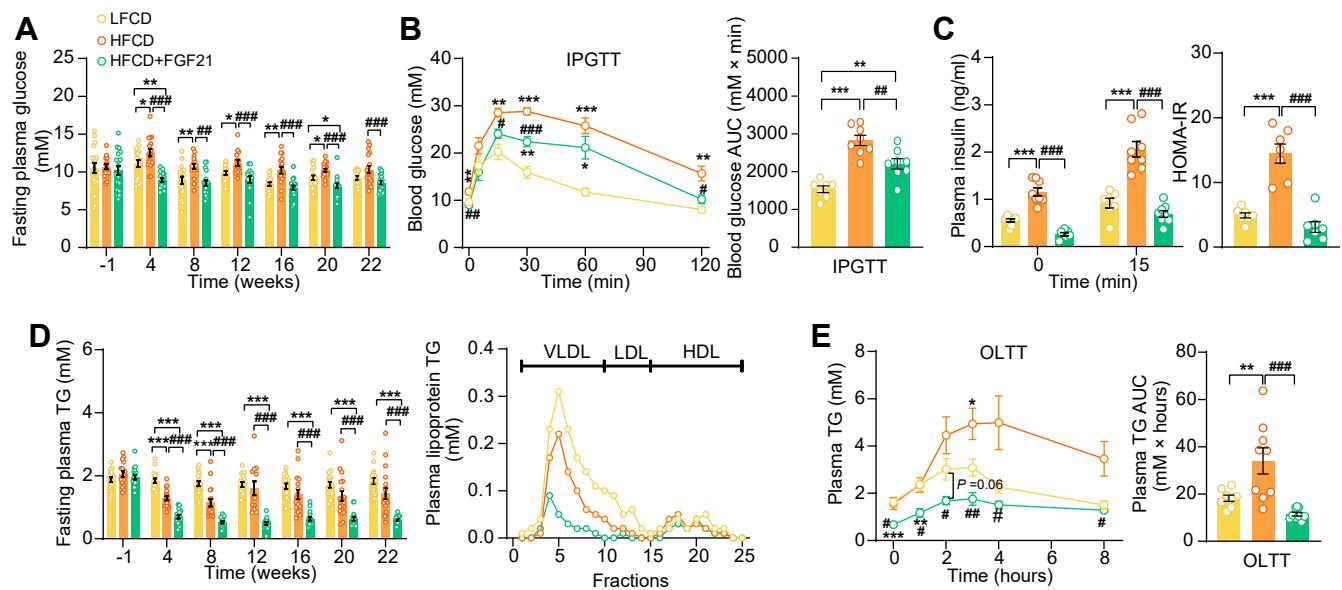
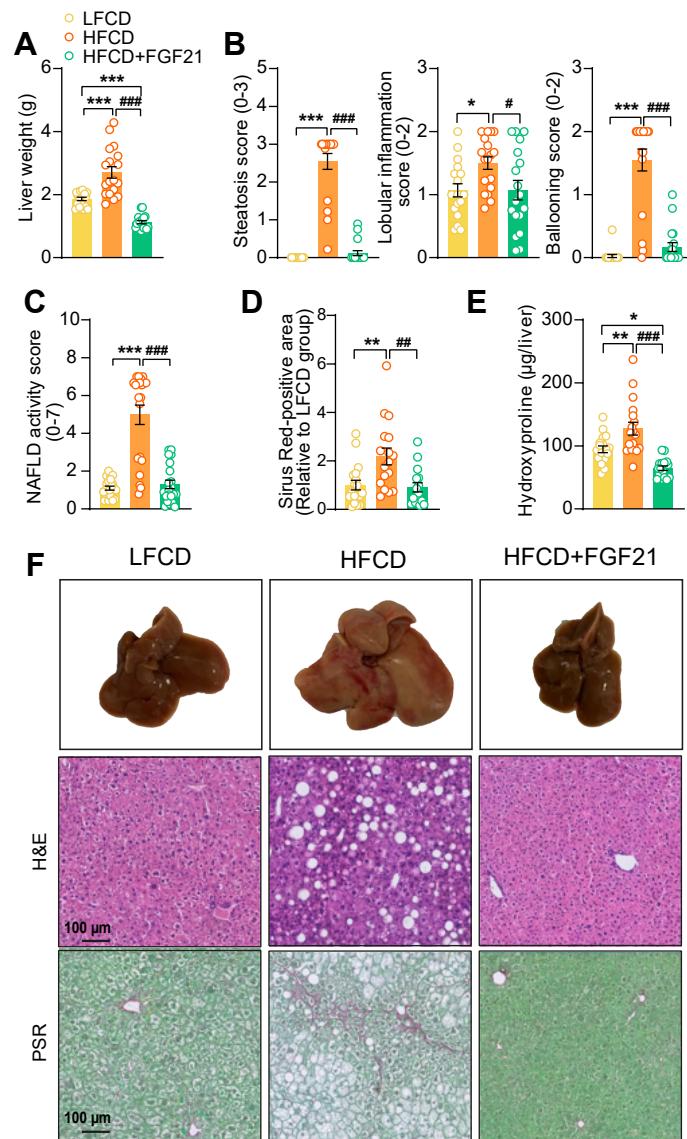


Figure 4



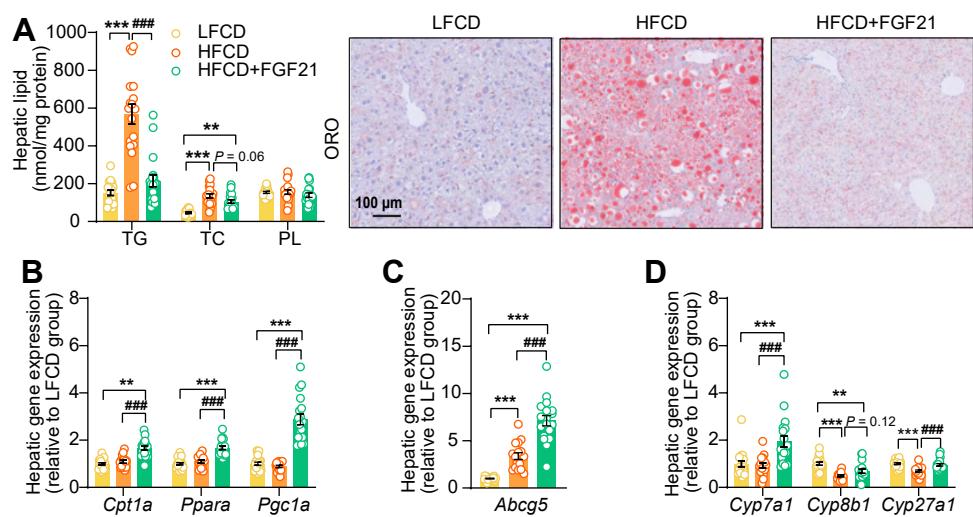


Figure 6

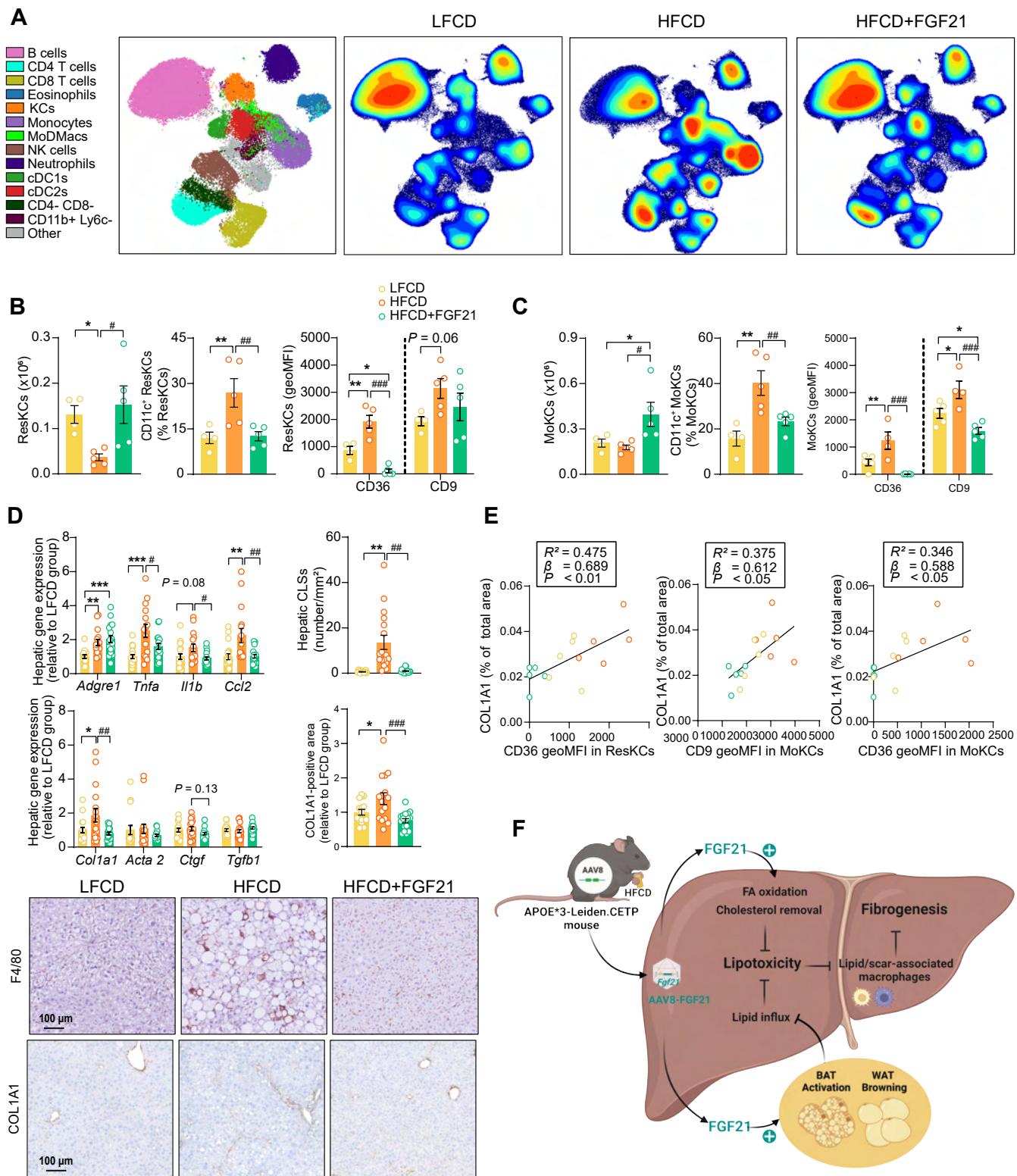
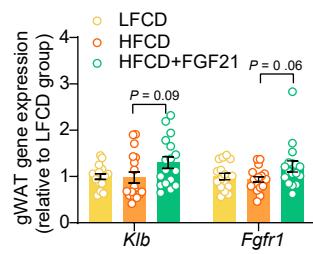
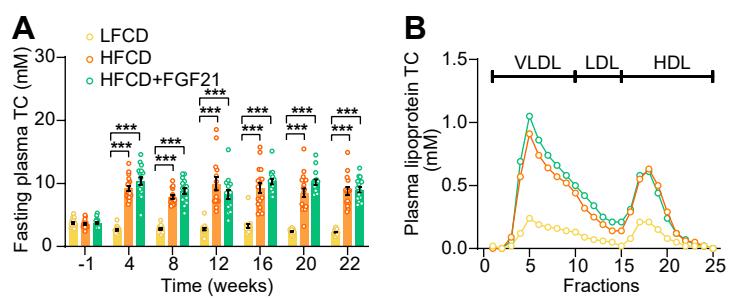


Figure 2 in the supplement





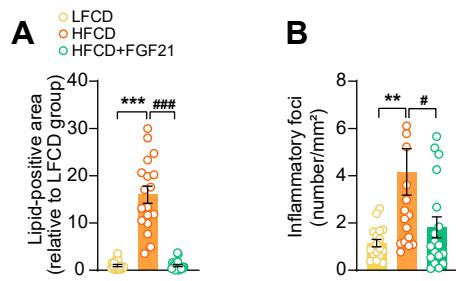
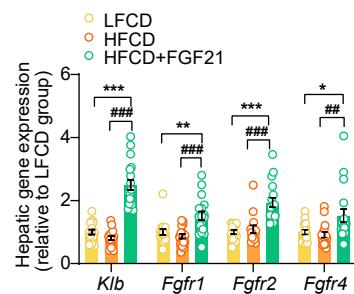
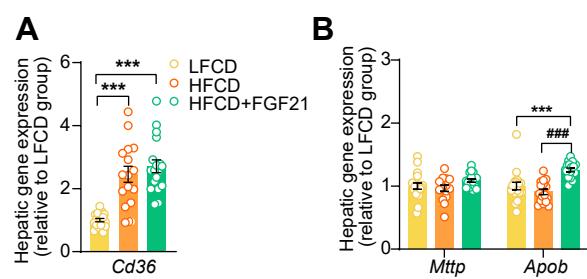


Figure 5 (more supplement)





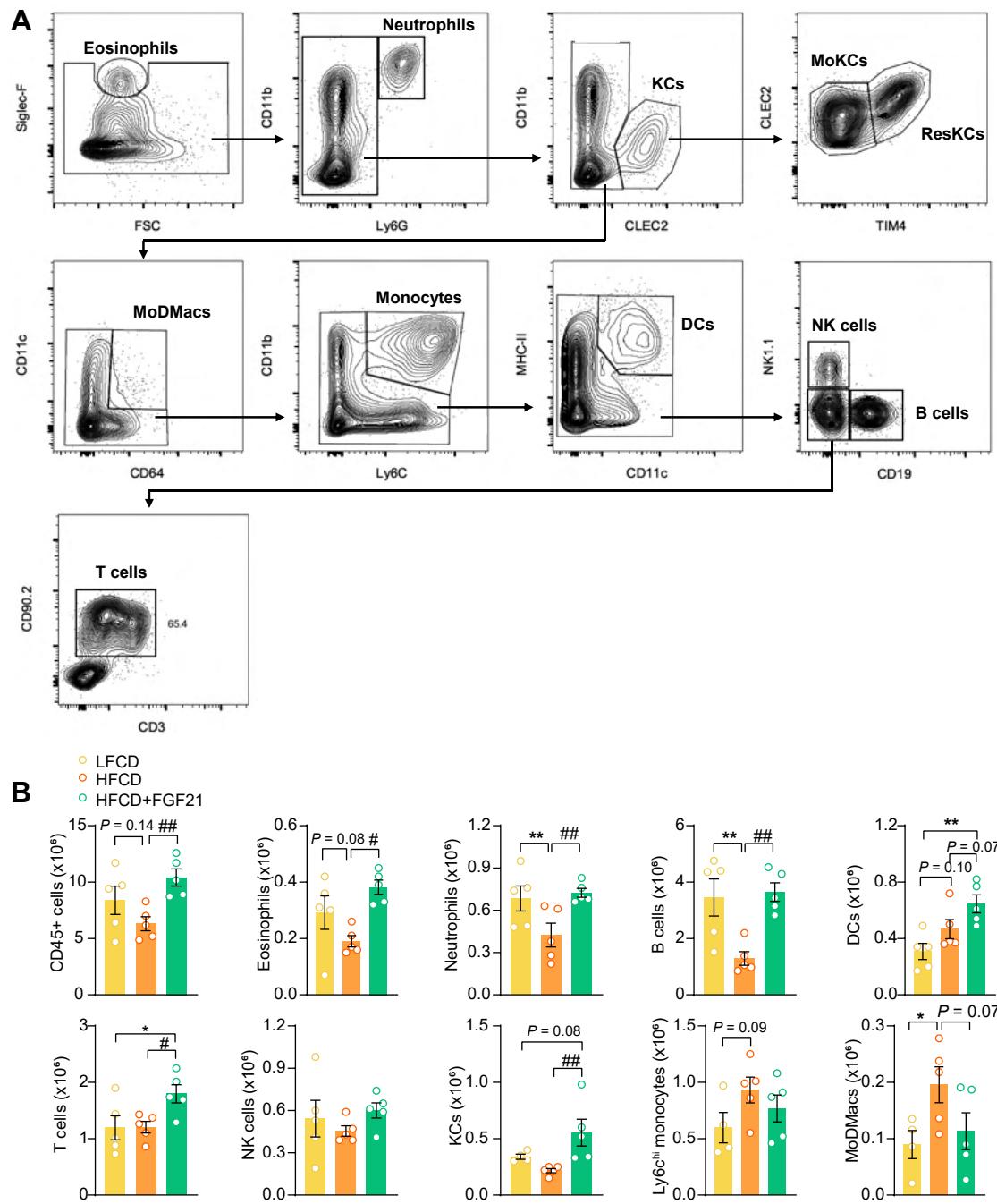


Figure 6 (figures supplement 2)

