

1 **GDF15 mediates the effects of metformin on body weight and energy balance**

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31 **Metformin, the world's most prescribed anti-diabetic drug, is also effective in**
32 **preventing Type 2 diabetes in people at high risk^{1,2}. Over 60% of this effect is**
33 **attributable to metformin's ability to lower body weight in a sustained manner³.**
34 **The molecular mechanisms through which metformin lowers body weight are**
35 **unknown. In two, independent randomised controlled clinical trials, circulating**
36 **levels of GDF15, recently described to reduce food intake and lower body weight**
37 **through a brain stem-restricted receptor, were increased by metformin. In wild-**
38 **type mice, oral metformin increased circulating GDF15 with *GDF15* expression**
39 **increasing predominantly in the distal intestine and the kidney. Metformin**
40 **prevented weight gain in response to high fat diet in wild-type mice but not in**
41 **mice lacking GDF15 or its receptor GFRAL. In obese, high fat-fed mice, the**
42 **effects of metformin to reduce body weight were reversed by a GFRAL**
43 **antagonist antibody. Metformin had effects on both energy intake and energy**
44 **expenditure that required GDF15. Metformin retained its ability to lower**
45 **circulating glucose levels in the absence of GDF15 action. In summary,**
46 **metformin elevates circulating levels of GDF15, which are necessary for its**
47 **beneficial effects on energy balance and body weight, major contributors to its**
48 **action as a chemopreventive agent.**

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54 Metformin has been used as a treatment for Type 2 diabetes since the 1950s. Recent
55 studies have shown that it can also prevent or delay the onset of Type 2 diabetes in
56 people at high risk^{1 2}. At-risk individuals treated with metformin manifest a reduction
57 in body weight, glucose and insulin levels and enhanced insulin sensitivity³. Although
58 many mechanisms for the insulin sensitizing actions of metformin have been proposed
59⁴, none would explain weight loss. The robustness and persistence metformin-induced
60 weight loss in participants in the Diabetes Prevention Program (DPP) has drawn
61 attention to the importance of this to the chemopreventive effects of the drug⁵. A
62 recent observational epidemiological study⁶ noted a strong association of metformin
63 use with circulating levels of GDF15, a peptide hormone produced by cells responding
64 to stressors⁷. GDF15 acts through a receptor complex solely expressed in the
65 hindbrain, through which it suppress food intake⁸⁻¹¹. We hypothesized that
66 metformin's effects to lower body weight might involve the elevation of circulating
67 levels of GDF15.

68 Human studies

69 We first measured circulating GDF15 in a short term human study¹² and found that
70 after 2 weeks of metformin, there was a ~2.5-fold increase in mean circulating
71 GDF15 (**Fig. 1a**).

72 To determine if this increase was sustained, we measured circulating GDF15 levels at
73 6, 12 and 18 months in all available participants in CAMERA¹³, a randomized placebo-
74 control trial of metformin in people without diabetes but with a history of cardiovascular
75 disease. In this study, metformin treated participants lost ~3.5% of body weight with
76 no significant change in weight in the placebo arm¹³. Metformin treatment was
77 associated with significantly ($p < 0.0001$) increased levels of circulating GDF15 at all

78 three time points (**Fig.1b** and **Extended Data Fig.1b,c,d,e**). Furthermore, the change
79 in serum GDF15 from baseline in metformin recipients was significantly correlated ($r=-$
80 0.26 , $p=0.024$) with weight loss (**Extended Data Fig. 1a**).

81 The correlation of GDF15 increment with changes in body weight, while statistically
82 significant, was modest in size. While we consider it does contribute to weight loss in
83 some individuals taking metformin, we acknowledge is by no means necessary and
84 there are individuals with increases in GDF-15 that do not exhibit weight loss.
85 However, in the context of a long term human study with imperfect drug compliance
86 and intermittent sampling of GDF15 levels it is noteworthy that such an association
87 was seen at all. Further, there was no association of weight change with change in
88 GDF-15 in the placebo group ($r=-0.0374$, $p=0.740$, $n=81$).”

89 Murine studies

90 Following these findings in humans, we undertook a series of animal experiments to
91 determine the potential causal link between the changes in GDF-15 and weight
92 changes induced by metformin. We administered metformin to high fat diet fed mice
93 by oral gavage and measured serum GDF15. A single dose of 300 mg/kg of metformin
94 increased GDF15 levels for at least 8 hours (**Fig. 1c**). A higher dose of metformin, 600
95 mg/kg, increased serum GDF15 levels 4-6 fold at 4 and 8-hours post-dose, which were
96 sustained over vehicle-treated mice for 24 hours. The effects of metformin in chow-
97 fed mice were less pronounced (**Extended Data Fig.2**) suggesting an interaction
98 between metformin and the high fat fed state.

99 To determine the extent to which metformin- induced increase in GDF15 affects body
100 weight, *Gdf15*^{+/+} and *Gdf15*^{-/-} mice were switched from chow to a high fat diet and
101 dosed with metformin for 11 days. High fat feeding induced similar weight gain in both

102 genotypes (**Fig. 2a**). Metformin completely prevented weight gain in *Gdf15*^{+/+} mice
103 but *Gdf15*^{-/-} mice were insensitive to the weight-reducing effects of metformin (**Fig.2a,**
104 **Extended data Fig.3a**). Metformin significantly reduced cumulative food intake in wild
105 type mice but this effect was abolished in *Gdf15*^{-/-} mice (**Fig. 2b**).

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107 The identical protocol was applied to mice lacking GFRAL, the ligand-binding
108 component of the hindbrain-expressed GDF15 receptor complex. Consistent with the
109 results in mice lacking GDF15, metformin was unable to prevent weight gain in *Gfral*⁻
110 ^{-/-} mice (**Fig. 2c, Extended data Fig.3b**), despite similar levels of serum GDF15
111 (**Extended Data Fig. 4a,b**). In this experiment, the reduction in cumulative food intake
112 did not reach statistical significance (**Extended Data Fig. 4c**).

113 To investigate the contribution of GDF15/GFRAL signalling to sustained, metformin-
114 dependent weight regulation, we performed a 9-week study in which mice received
115 approximately 250-300 mg/kg/day of metformin incorporated into their high-fat diet.
116 The mice lost ~10% body weight after 1 month on this diet (**Fig. 2d**). At this time, an
117 anti-GFRAL antagonist antibody or IgG control was administered. Metformin-
118 consuming mice treated with anti-GFRAL regained ~12% body weight after 5 weeks,
119 while the weight loss seen in IgG control treated mice was -maintained, reaching
120 ~7% below starting weight (**Fig. 2d**). The significant reduction in fat mass seen with
121 metformin treatment and control antibody was not seen in the anti-GFRAL group.
122 (**Extended Data Fig. 4d**). **The delivery of metformin in chow resulted in an initial**
123 **reduction in food intake in all metformin treated groups, presumably because of a**
124 **taste effect. This reduction in food intake will have affected metformin levels and is**
125 **likely to have impacted GDF15 levels with potential to bias the results. However, it is**

126 reassuring to note that any persistence of this would have worked against the
127 detection of a specific effect of GFRAL antagonism, which was clearly demonstrable.

128 We undertook indirect calorimetry in metformin- and placebo-treated mice treated with
129 anti-GFRAL antibody to establish whether there are additional effects on energy
130 expenditure. Data were analysed by ANCOVA with body weight as the co-variate.
131 Metformin treatment resulted in a significant increase in metabolic rate which was
132 blocked by antagonism of GFRAL (**Fig. 2e**). Thus under conditions where GDF15
133 levels are increased by metformin, body weight reduction is contributed to by both
134 reduced food intake and an inappropriately high energy expenditure.

135 **GDF15 and -glucose homeostasis**

136 To examine the extent to which the insulin sensitising effects of metformin are
137 dependent on GDF15 we repeated the experiment described in **Fig.2a** (see **Extended**
138 **Data Fig. 5**), undertaking insulin tolerance testing in metformin and vehicle-treated
139 GDF15 null mice and their wild type littermates (**Fig. 3a**). Circulating metformin levels
140 achieved in both genotypes were identical (**Extended Data Fig. 5d**) and consistent
141 with the high end of the human therapeutic range¹⁴. Metformin significantly increased
142 insulin sensitivity as assessed by the area under the plasma glucose curve with no
143 significant effect of genotype (**Fig. 3b**). Similarly, metformin reduced fasting blood
144 glucose and fasting insulin in a GDF15-independent manner (**Fig. 3 c,d**).

145 We also undertook oral glucose tolerance testing of metformin treated mice given
146 either control IgG or anti-GFRAL antibody for 5 weeks (**Fig 3e,f, Extended Data Fig.**
147 **6a** and see **Fig. 2d**). Although the effect of metformin glucose disposal at OGTT as
148 assessed by the area under the plasma glucose curve did not reach statistical
149 significance (2W ANOVA, $p=0.072$), there was a significant effect of metformin on

150 insulin, both fasting and AUC after glucose bolus, that was independent of antibody
151 **(Fig. 3 g,h,i,j).**

152 As these mice were of different body weight at the time of assessment (**Fig. 2d** and
153 **Extended Data Fig. 3c**), we undertook further glucose tolerance testing in a cohort
154 of weight matched *Gdf15^{+/+}* and *Gdf15^{-/-}* mice that had been fed a high fat diet for 2
155 weeks before receiving a single dose of metformin (300mg/kg) (**Fig 3k,l** and **Extended**
156 **Data Fig. 6b-d**) In these mice there was a significant effect of metformin upon glucose
157 (AUC plasma glucose) that was independent of GDF15 (extended Data Fig. 6 e).

158 Metformin's effect to lower fasting glucose and insulin and to improve glucose
159 tolerance appear not to require GDF15. Given the "a priori" expected effect of weight
160 loss on insulin sensitivity it is worthy of comment that the effect of GDF15 status on
161 insulin sensitivity as measured by ITT (**Fig 3b**) fell just short of statistical
162 significance. In the follow up of the DPP study in non-diabetic individuals, weight
163 loss after 5 years of metformin therapy was approximately 6.5% of baseline weight⁵ .
164 We therefore estimated the effect of a 6.5% weight loss on improvements in fasting
165 insulin over 5 years in the Ely Study, a prospective observational population-based
166 cohort study of men (n=465) and women (n=634) in the UK (mean age 52 years,
167 mean BMI 26 at baseline)¹⁵ , showing that this magnitude of weight loss was
168 associated with a reduction in fasting plasma insulin (mean \pm 95% CI) of -5.74 (-
169 9.03, -2.45) pmol/l in women and -8.78 (-16.24, -1.33) in men. We conclude that
170 while there are GDF15-independent effects of metformin on circulating levels of
171 glucose and insulin, it is likely that the GDF15 dependent weight loss will make a
172 contribution to enhancing insulin sensitivity.

173

174 Source of GDF15 production

175 We examined GDF15 gene expression in a tissue panel obtained from mice fed a high
176 fat diet (for 4 weeks) and sacrificed 6 hours after a single gavage dose of metformin
177 (600mg/kg). Circulating concentrations of GDF15 increased ~4-fold compared to
178 vehicle treated mice (**Extended Data Fig. 6f**). *Gdf15* mRNA was significantly
179 increased by metformin in small intestine, colon and kidney. (**Fig. 4a**). In situ
180 hybridisation studies demonstrated strong *Gdf15* expression in crypt enterocytes in
181 the colon and small intestine and in periglomerular renal tubular cells (**Fig. 4b**,
182 **Extended Data Fig. 7a, b**). We confirmed these sites of tissue expression in HFD fed
183 mice (those used in **Fig 2a**), treated with metformin for 11 days (**Extended Data Fig.**
184 **8**).

185 Further, in human (**Fig. 4c**) and murine (**Fig. 4d**) intestinal-derived organoids grown
186 in 2D transwells and treated with metformin, we saw a significant induction of mRNA
187 expression and GDF15 protein secretion.

188 Given the proposed importance of the liver for metformin's metabolic action it was
189 notable that the dominant GDF15 expression signal was not from the liver (**Fig. 4a**,
190 **Extended Data Fig. 7a, Extended Data Fig. 8**). To test whether hepatocytes are
191 capable of responding to biguanide drugs with an increase in GDF15 we incubated
192 freshly isolated murine hepatocytes (**Extended Data Fig. 9a**) and stem-cell derived
193 human hepatocytes (**Extended Data Fig. 9b**) with metformin and found a clear
194 induction of GDF15 expression. Additionally, acute administration of the more cell
195 penetrant biguanide drug phenformin to mice increased circulating GDF15 levels
196 (**Extended Data Fig. 9c**) and markedly increased *Gdf15* mRNA expression in
197 hepatocytes (**Extended Data Fig. 9d,e**). We conclude that biguanides can induce

198 GDF15 expression in many cell types, but at least when given orally to mice, GDF15
199 mRNA is most strikingly induced in the distal small intestine, colon and kidney.

200 GDF15 expression has been reported to be a downstream target of the cellular
201 integrated stress response (ISR) pathway¹⁶⁻¹⁸. *Gdf15* mRNA levels were increased in
202 kidney and colon 24 h after a single oral dose of metformin and these changes
203 correlated positively with the fold elevation of CHOP mRNA (**Extended Data Fig.**
204 **10a,b**). As phenformin has broader cell permeability than metformin¹⁹ we used it to
205 explore the effects of biguanides on the ISR and its relationship to GDF15 expression
206 in cells. In murine embryonic fibroblasts (MEFs), which do not express the organic
207 cation transporters needed for the uptake of metformin, phenformin (but not
208 metformin) increased EIF2 α phosphorylation, ATF4 and CHOP expression,
209 (**Extended Data Fig. 10c**) and GDF15 mRNA (**Extended Data Fig. 10d**), though the
210 changes in EIF2a phosphorylation and ATF4 and CHOP expression were modest
211 compared with those induced by tunicamycin despite similar levels of GDF15 mRNA
212 induction. Both genetic deletion of ATF4 and siRNA-mediated knockdown of CHOP
213 significantly reduced phenformin-mediated induction of GDF15 mRNA expression
214 (**Extended Data Fig. 10e,f**). In addition, phenformin induction of GDF15 was markedly
215 reduced by co-treatment with the EIF2 α inhibitor, ISRIB but, notably, not by the PERK
216 inhibitor, GSK2606414 (**Extended Data Fig. 10g**). Further, GDF15 secretion in
217 response to metformin in murine duodenal organoids was also significantly reduced
218 by co-treatment with ISRIB (**Extended Data Fig. 10h**). However, gut organoids
219 derived from CHOP null mice are still able to increase GDF15 secretion in response
220 to metformin (**Extended Data Fig. 10i**) indicating the existence of CHOP-independent
221 pathways under some circumstances. The data suggest that the effects of biguanides
222 on GDF15 expression are at least partly dependent on the ISR pathway but are

223 independent of PERK. However, the relative importance of components of the ISR
224 pathway may vary depending on specific cell type, dose and agent used.

225 Our observations represent a significant advance in our understanding of the action of
226 metformin, one of the world's most frequently prescribed drugs. Metformin increases
227 circulating GLP1 levels²⁰⁻²² , but its metabolic effects in mice are unimpaired in mice
228 lacking the GLP-1 receptor ²³. Metformin alters the intestinal microbiome^{24,25} but it is
229 challenging to firmly establish a causal relationship to the beneficial effects of the drug
230 ²⁶.

231 In the work presented herein, we describe a body of data from humans, cells,
232 organoids and mice that securely establish a major role for GDF15 in the mediation
233 of metformin's beneficial effects on energy balance. While these effects likely
234 contribute to metformin's role as an insulin sensitizer, metformin continues to have
235 effects to lower glucose and insulin in the absence of GDF15.

236

237 While there have been many mechanisms suggested for the glucoregulatory
238 mechanisms of metformin²⁷ there has been less attention paid to its effects on
239 weight. Our discoveries relating to metformin's effects via GDF15 provide a
240 compelling explanation for this important aspect of metformin action.

241 It is notable that the lower small intestine and colon are a major site of metformin
242 induced GDF15 expression. A body of work is emerging which strongly implicates
243 the intestine as a major site of metformin action. Metformin increased glucose uptake
244 into colonic epithelium from the circulation²⁸ and a gut-restricted formulation of
245 metformin had greater glucose lowering efficacy than systemically absorbed
246 formulations ²⁹. Our finding that the intestine is a major site of metformin-induced

247 GDF15 expression provides a further mechanism through which metformin's action
248 on the intestinal epithelium may mediate some of its benefits.

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328 **Figure Legend**

329 **Figure 1. Effect of Metformin on circulating GDF15 levels in humans and mice.**

330 **a**, Paired serum GDF15 concentration in 9 human subjects after 2 weeks of either
331 placebo or metformin, P (95% confidence interval) by 2-tailed t-test.

332 **b**, Plasma GDF15 concentration (mean± SEM) in overweight or obese non-diabetic
333 participants with known cardiovascular disease randomised to metformin or placebo
334 in CAMERA, using a mixed linear model. Subject numbers: placebo vs metformin,
335 respectively, at time points: baseline, n=85 vs n=86; 6 months, n=81 vs n= 71; 12
336 months, n=77 vs n=68; 18 months, n=83 vs n=74. Comparing metformin vs placebo
337 groups, two-sided p=0.311 at baseline, and p<0.0001 at 6,12 and 18 months
338 individually.

339 **c**, Serum GDF15 levels (mean± SEM) in obese mice measured 2, 4, 8 or 24 hours
340 after a single oral dose of 300 mg/kg or 600 mg/kg metformin, n=7/group, P by 2-way
341 ANOVA with Tukey's correction for multiple comparisons.

342

343 **Figure 2. GDF15/GFRAL signalling is required for the weight loss effects of**
344 **metformin on a high fat diet.**

345 **a**, Percentage change in body weight of Gdf15+/+ and Gdf15-/- mice on a high-fat
346 diet treated with metformin (300mg/kg/day) for 11 days, mean ± SEM, n=6/group
347 except Gdf15+/+ vehicle n=7, P by 2-way ANOVA with Tukey's correction for
348 multiple comparisons.

349 **b**, Cumulative food intake of mice as Figure 2a, P by 2-way ANOVA with Tukey's
350 correction for multiple comparisons.

351 **c**, Percentage change in body weight of Gfral+/+ and Gfral-/- mice on a high-fat diet
352 treated with metformin (300mg/kg/day) for 11 days, mean ± SEM, n=6/groups, P by
353 2-way ANOVA with Tukey's correction for multiple comparisons.

354 **d**, Percentage change in body weight of metformin-treated obese mice dosed with
355 an anti-GFRAL antagonist antibody, weekly for 5 weeks (yellow), starting 4 weeks
356 after initial metformin exposure (grey), mean ± SEM, n=7 Vehicle + control IgG and
357 Metformin + anti -GFRAL, n=8 other groups, P by 2-way ANOVA with Tukey's
358 correction for multiple comparisons. "calo" = period in which energy expenditure
359 measured (see Figure 2e), Arrow and "GTT"- timing of oral glucose tolerance test
360 (see Figure 3e-h).

361 **e**, ANCOVA analysis of energy expenditure against body weight of mice treated as in
362 Figure 2d, n=6 mice/group. Data are individual mice and P for metformin calculated
363 using ANCOVA with body weight as a covariate and treatment as a fixed factor.

364

365 **Figure 3. Effects of metformin on glucose homeostasis.**

366 **a**, Insulin tolerance test (ITT) (insulin=0.5 U/kg) after 11 days of metformin treatment
367 (300mg/kg) to high fat fed Gdf15 +/+ and Gdf15 -/- mice, glucose levels are mean ±
368 SEM, n=6/group, except Gdf15 -/- vehicle= 7, Gdf15+/+ vehicle= 5.

369 **b**, Area under curve (AUC) analysis of glucose over time in Figure 3a, mean ± SEM,
370 P by 2-way ANOVA , interaction of genotype and metformin p= 0.037.

371 **c**, Fasting glucose (time 0) of ITT in Figure 3a, mean ± SEM, P by 2-way ANOVA,
372 effect of genotype p= 0.144, interaction of genotype and metformin p= 0.988.

373 **d**, Fasting insulin (time 0) of ITT in Figure 3a, mean ± SEM, P by 2-way ANOVA,
374 effect of genotype p= 0.131, interaction of genotype and metformin p 0.056.

375 **e, f**, Glucose over time after oral glucose tolerance test (GTT) in metformin treated
376 obese mice given either IgG (e) or anti -GFRAL (f) once weekly for 5 weeks (as

377 Figure 2d). AUC analysis by 2-way ANOVA, effect of antibody $p= 0.031$, effect of
378 metformin $p= 0.072$, interaction of antibody and metformin $p 0.91$.

379 **g, h**, Insulin (mean \pm SEM) over time after GTT in mice as Figure 3e and f.

380 **i**, Fasting insulin (time 0) of GTT in mice as Figure 3e and f, mean \pm SEM, P by 2-
381 way ANOVA, effect of antibody $p= 0.544$, interaction of genotype and metformin p
382 0.691 .

383 **j**, AUC analysis of insulin over time in Figure 3g and h, mean \pm SEM, P by 2 -way
384 ANOVA, effect of antibody $p= 0.197$, interaction of genotype and metformin $p 0.607$.

385 **k, l**, Glucose (mean \pm SEM) over time after intraperitoneal GTT in high fat fed mice
386 given single dose of oral metformin (300mg/kg) 6 hrs before GTT, $n=8$ /group.

387

388 **Figure 4. Metformin increases GDF15 expression in the enterocytes of distal**
389 **intestine and the renal tubular epithelial cells.**

390 **a**, Gdf15 mRNA expression (normalised to expression levels of ActB) in tissues from
391 high-fat fed wild type mice 6 hrs after single dose of oral metformin (600mg/kg),
392 mean \pm SEM, $n=7$ /group, P value (95% confidence interval) by two tailed t-test.

393 **b**, In situ hybridization for Gdf15 mRNA (red spots) $n= 7$ per group. Representative
394 images from the mouse with circulating GDF15 level closest to group median, either
395 vehicle-treated (panel 1a,1b,1c, blue box) or metformin-treated (panels 2a, 2b, 2c,
396 red box). Mice from groups described in Figure 4a.

397 **c**, Gdf15 mRNA expression (left panel) and GDF15 protein in supernatant (right
398 panel) of human derived 2D monolayer rectal organoids treated with metformin.
399 Each colour represents independent experiments ($n= 4$), mean \pm SD, P value (95%
400 confidence interval) by two-tailed t-test.

401 **d**, GDF15 protein in supernatants of mouse-derived 2D monolayer duodenal (left
402 panel) and ileal (right panel) organoids treated with metformin. Each colour
403 represents independent experiment (duodenal $n= 5$, ileal $n=3$), mean \pm SD, P value
404 (95% confidence interval) by two-tailed t-test.

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411 **Methods.**

412 **Human Studies.**

413 We analysed samples from 9 participants from a study with a placebo-controlled,
414 double-blind crossover design (previously described in¹²). In brief, placebo or
415 metformin (week 1, 500mg twice daily; week, 2 1000mg twice daily) were
416 administered following a six week period of washout. Samples were collected in the
417 morning after overnight fasting. The study was approved by the Mayo Clinic
418 Institutional Review Board and all participants provided written, informed consent
419 (NCT01956929).

420 CAMERA was a randomized, double-blinded, placebo-controlled trial designed to
421 investigate the effect of metformin on surrogate markers of cardiovascular disease in
422 patients without diabetes, aged 35 to 75, with established coronary heart disease
423 and a large waist circumference (≥ 94 cm in men, ≥ 80 cm in women)
424 (NCT00723307). This single-centre trial enrolled 173 adults who were followed up for
425 18 months each. A detailed description of the trial and its results has been published
426 previously¹³. In brief, participants were randomized 1:1 to 850mg metformin or
427 matched placebo twice daily with meals. Participants attended six monthly visits after
428 overnight fasts and before taking their morning dose of metformin. Blood samples
429 collected during the trial were centrifuged at 4 degrees Celsius soon after sampling,
430 separated and stored at -80°C

431 All participants provided written informed consent. The study was approved by the
432 Medicines and Healthcare Products Regulatory Agency and West Glasgow
433 Research Ethics Committee, and done in accordance with the principles of the
434 Declaration of Helsinki and good clinical practice guidelines.

435 Serum GDF15 assays were completed by the Cambridge Biochemical Assay
436 Laboratory, University of Cambridge. Measurements were undertaken with
437 antibodies & standards from R&D Systems (R&D Systems Europe, Abingdon UK)
438 using a microtiter plate-based two-site electrochemiluminescence immunoassay
439 using the MesoScale Discovery assay platform (MSD, Rockville, Maryland, USA).

440 **Mouse Studies.**

441 Studies were carried out in two sites; NGM Biopharmaceuticals, California, USA and
442 University of Cambridge, UK.

443 At NGM, all experiments were conducted with NGM IACUC approved protocols and
444 all relevant ethical regulations were complied with throughout the course of the
445 studies, including efforts to reduce the number of animals used. Experimental
446 animals were kept under controlled light (12hour light and 12hour dark cycle, dark
447 6:30 pm - 6:30 am), temperature ($22 \pm 3^{\circ}\text{C}$) and humidity ($50\% \pm 20\%$) conditions.
448 They were fed ad libitum on 2018 Teklad Global 18% Protein Rodent Diet containing
449 24 kcal% fat, 18 kcal% protein and 58 kcal% carbohydrate, or on high fat rodent diet
450 containing 60 kcal% fat, 20 kcal% protein and 20 kcal% carbohydrates from
451 Research Diets D12492i,(New Brunswick NJ 089901 USA) herein referred to as
452 “60%HFD”.

453 In Cambridge, all mouse studies were performed in accordance with UK Home
454 Office Legislation regulated under the Animals (Scientific Procedures) Act 1986
455 Amendment, Regulations 2012, following ethical review by the University of
456 Cambridge Animal Welfare and Ethical Review Body (AWERB). They were
457 maintained in a 12-hour light/12-hour dark cycle (lights on 0700–1900),
458 temperature-controlled (22°C) facility, with ad libitum access to food (RM3(E)

459 Expanded chow, Special Diets Services, UK) and water. Any mice bought from an
460 outside supplier were acclimatised in a holding room for at least one week prior to
461 study. During study periods they were fed ad libitum high fat diet, either D12451i (45
462 kcal% fat, 20 kcal% protein and 35 kcal% carbohydrates, herein referred to as
463 “45%HFD”) or D12492i (Research Diets, as above) as highlighted in individual
464 study.

465 Sample sizes were determined on the basis of homogeneity and consistency of
466 characteristics in the selected models and were sufficient to detect statistically
467 significant differences in body weight, food intake and serum parameters between
468 groups. Experiments were performed with animals of a single gender in each study.
469 Animals were randomized into the treatment groups based on body weight such that
470 the mean body weights of each group were as close to each other as possible, but
471 without using excess number of animals. No samples or animals were excluded from
472 analyses. Researchers were not blinded to group allocations.

473 **Mouse study 1. Acute two- dose metformin study in high fat diet fed mice.**

474 Male C57Bl6/J mice fed 60% HFD for 17 weeks were studied aged 23 weeks (body
475 weight, mean \pm SEM, 45.6 \pm 0.8g). Metformin (Sigma-Aldrich # 1396309) was
476 reconstituted in water at 30 mg/ml for oral gavage and given in early part of light
477 cycle. Terminal blood was collected by cardiac puncture into EDTA- coated tubes.
478 GDF15 levels were measured using Mouse/Rat GDF15 Quantikine ELISA Kit (Cat#:
479 MGD-150, R&D Systems, Minneapolis, MN) according to the manufacturers'
480 instructions. RNA was isolated from tissues using the Qiagen RNeasy Kit. RNA was
481 quantified and 500ng was used for cDNA synthesis (SuperScript VILO 11754050
482 ThermoFisher) followed by qPCR. All Taqman probes were purchased from Applied

483 Biosystems. All genes are expressed relative to 18s control probe and were run in
484 triplicate.

485

486 **Mouse study 2. Acute metformin study in chow fed animals.**

487 **2.i) ad libitum group.**

488 Male C57BL6/J mice (Charles River, Margate, UK) were studied at 11 weeks old.
489 500mg of metformin was dissolved in 20 mls of water to make a working stock of
490 25mg/ml. 1 hr after onset of light cycle mice received a single dose by oral gavage
491 of either metformin at 300mg/kg dose (Sigma, PHR1084-500MG) or matched
492 volume of vehicle (water). Weight (mean \pm SEM) of control and treatment groups
493 were 27.2 \pm 0.3 vs 26.7 \pm 0.2 g, respectively on day of study. After gavage mice
494 were returned to an individual cage and were sacrificed at relevant time point by
495 terminal anaesthesia (Euthatal by Intraperitoneal injection). Blood was collected
496 into Sarstedt Serum Gel 1.1ml Micro Tube, left for 30mins at room temperature,
497 spun for 5mins at 10k at 40C before being frozen and stored at -80oC until assayed.
498 Mouse GDF15 levels were measured using a Mouse GDF15 DuoSet ELISA (R&D
499 Systems) which had been modified to run as an electrochemiluminescence assay on
500 the Meso Scale Discovery assay platform.

501 **2.ii) fasted group.**

502 Mice, conditions and methods as in (2.i) except male mice studied at 9 weeks old
503 and that 12 hr prior to administration of metformin mice and bedding were
504 transferred to new cages with no food in hopper. Weight (mean \pm SEM) after fasting
505 and on day of gavage were 22.3 \pm 0.5 g and 23.2 \pm 0.7g for control and treatment
506 groups, respectively.

507 **Mouse study 3. Metformin to high fat diet fed *Gdf15*^{-/-} mice and wild type**
508 **controls.**

509 C57BL/6N-Gdf15^{tm1a(KOMP)}Wtsi/H mice (herein referred to as “Gdf15^{-/-} mice”)
510 were obtained from the MRC Harwell Institute which distributes these mice on behalf
511 of the European Mouse Mutant Archive (www.infrafrontier.eu). The MRC Harwell
512 Institute is also a member of the International Mouse Phenotyping Consortium
513 (IMPC) and has received funding from the MRC for generating and/or phenotyping
514 the C57BL/6N-Gdf15^{tm1a(KOMP)}Wtsi/H mice. The research reported in this
515 publication is solely the responsibility of the authors and does not necessarily
516 represent the official views of the Medical Research Council. Associated primary
517 phenotypic information may be found at www.mousephenotype.org. Details of the
518 alleles have been published³⁰⁻³².

519 Experimental cohorts of male *Gdf15*^{-/-} and wild type mice were generated by het x
520 het breeding pairs. Mice were aged between 4.5 and 6.5 months. One week prior to
521 study start mice were single housed and 3 days prior to first dose of metformin
522 treatment, mice were transferred from standard chow to 60% high fat diet. On day of
523 first gavage body weight of study groups (mean±SEM) were 38.2±1.0g vs 38.8±0.6g
524 for wild type vehicle and metformin treatment respectively, and 37.9±0.8g vs
525 37.0±1.4g for *Gdf15*^{-/-} vehicle and metformin treatment respectively. Each mouse
526 received a daily gavage of either vehicle or metformin for 11 days, and their body
527 weight and food intake measured daily in the early part of the light cycle. One data
528 point of 25 food intake points collected on day11 of study was lost due to technical
529 error (mouse; *Gdf15*^{+/+} metformin). On day 11 mice were sacrificed by terminal
530 anaesthesia 4 hours post gavage, blood was obtained as in study 2. Tissues were
531 fresh frozen on dry ice and kept at -80°C until day of RNA extraction.

532

533 **Mouse study 4. Metformin to high fat diet fed *Gfral*^{-/-} mice.**

534 *Gfral*^{-/-} mice were purchased from Taconic (#TF3754) on a mixed 129/SvEv-C57BL/6
535 background and backcrossed for 10 generations to >99% C57BL/6 background at
536 NGM's animal facility. Experimental cohorts were generated by het X het breeding
537 pairs. Study design as Study 3, except terminal blood was collected into EDTA-
538 coated tubes.

539 **Mouse study 5. Anti GFRAL antibody to metformin treated high fat diet fed**
540 **mice.**

541 **Anti-GFRAL antibody generation.** Anti-GFRAL monoclonal antibodies were
542 generated by immunizing C57Bl/6 mice with recombinant purified GFRAL ECD-hFc
543 fusion protein, which was purified via sequential protein-A affinity and size exclusion
544 chromatography (SEC) techniques using MabSelect SuRe and Superdex 200
545 purification media respectively (GE Healthcare), as described in patent number
546 US10174119B2, <https://patents.google.com/patent/US10174119B2/en>. An in-house
547 pTT5 hlgK hlgG1 expression vector was engineered to include the DEVDG
548 (caspase-3) proteolytic site N-terminal to the Fc domain. The heavy chains of anti-
549 GFRAL mAbs were subcloned via EcoR1/HindIII sites of in-house engineered pTT5
550 hlgK hlgG1 caspase-cleavable vector. Light chains of anti-GFRAL mAbs were also
551 subcloned within the EcoR1/HindIII sites in the pTT5 hlgK hKappa vector. The
552 antibody were transiently expressed in Expi293 cells (Thermo Fisher Scientific)
553 transfected with the pTT5 expression vector, and purified from conditioned media by
554 sequential protein-A affinity and size-exclusion chromatographic (SEC) methods
555 using MabSelect SuRe and Superdex 200 purification media respectively (GE

556 Healthcare). All purified antibody material was verified endotoxin-free and formulated
557 in PBS for in vitro and in vivo studies. Characterization of anti-GFRAL functional
558 blocking antibodies was carried out using a cell-based RET/GFRAL luciferase gene
559 reporter assays, in vitro binding studies (ELISA and Biacore) and in vivo studies, as
560 described in patent number; US10174119B2,
561 <https://patents.google.com/patent/US10174119B2/en>).

562 In all studies with anti-GFRAL, purified recombinant non-targeting IgG on the same
563 antibody framework was used as control. Metformin was mixed with food paste
564 made from the 60 kcal% fat diet (Research diet# D12492) using a food blender at a
565 concentration to achieve an approximate consumption of 300mg/kg metformin per
566 day per mouse. Male animals were single housed throughout and at start of study
567 period body weight (mean \pm SEM) was 43.7 \pm 1.4g, 42.3 \pm 1.4g, 41.9 \pm 1.1g,43.3 \pm 1.3g,
568 veh + control IgG, veh +anti-GFRAL, metformin + control IgG, Metformin + anti-
569 GFRAL, respectively. Recombinant antibodies were administered by subcutaneous
570 injection in the early part of the light cycle. Body composition (lean and fat mass)
571 was analyzed by ECHO MRI M113 mouse system (Echo Medical Systems). The
572 metabolic parameters oxygen consumption (VO₂) and carbon dioxide production
573 (VCO₂) were measured by an indirect calorimetry system (LabMaster TSE System,
574 Germany) in open circuit sealed chambers. Measurements were performed for the
575 dark (from 6pm to 6am) or light (from 6am to 6pm) period under ad libitum feeding
576 conditions. Mice were placed in individual metabolic cages and allowed to acclimate
577 for a period of 24 hours prior to data collection in every 30 minutes.

578 Finally, mice underwent a glucose tolerance test. Mice were fasted for 6 hours
579 (7am-1pm) in a clean cage. Blood samples (~30 ul) were collected as baseline prior
580 to oral glucose tolerance test. Mice were orally gavaged with 1 g/kg of 20% glucose

581 solution with a dosing volume of 5 mL/kg. Blood samples were then collected
582 through tail nick into K2EDTA-coated tubes (SARSTEDT Microvette; REF
583 20.1278.100) at 15, 30, 60 and 120 minutes post glucose challenge. Blood samples
584 were centrifuged at 4 °C and the separated plasma are stored at -20 °C until used
585 for plasma glucose and insulin assays. Glucose assay reagents obtained from
586 Wako, Cat# 439-90901, and the insulin ELISA kit obtained from ALPCO, Cat# 80-
587 INSMSU-E01.

588

589 **Mouse study 6. Insulin tolerance test after metformin treatment to high fat diet**
590 **fed *Gdf15*^{-/-} and wild type controls.**

591 Mice generation and protocol as Study 3, except aged 4 to 6 months. On day of first
592 gavage body weights (mean±SEM) of study groups were 35.1±1.2g; 35.05±1.2g for
593 wild type Vehicle and Metformin treatment respectively, and 35.08±1.02g;
594 35.02±1.47g for *Gdf15*^{-/-} Vehicle and Metformin treatment respectively. On day 11,
595 after final dose of metformin mice were fasted for 4 hours. Baseline venous blood
596 sample was collected into heparinised capillary tube for insulin measurement and
597 blood glucose was measured using approximately 2 µl blood drops using a
598 glucometer (AlphaTrak2; Abbot Laboratories) and glucose strips (AlphaTrak2 test 2
599 strips, Abbot Laboratories, Zoetis) .Mice were given intraperitoneal injection of insulin
600 (0.5U/kg mouse, Actrapid, NovoNordisk Ltd) and serial mouse glucose levels
601 measured at time points indicated. Mice were sacrificed by terminal anaesthesia as
602 in Study 2. Mouse insulin was measured using a 2-plex Mouse Metabolic
603 immunoassay kit from Meso Scale Discovery Kit (Rockville, MD, USA), performed
604 according to the manufacturer's instructions and using calibrators provided by MSD.

605 Serum metformin levels were quantified using a stable isotope dilution LC-MS/MS
606 method described previously³³ .

607 **Mouse study 7. Glucose tolerance test after single dose metformin treatment**
608 **to high fat diet fed Gdf15^{-/-} and wild type controls.**

609 Mice generation as Study 3, except female mice aged 3.5 to 5.5 months. 2 groups of
610 mice (*Gdf15^{+/+}* and *Gdf15^{-/-}* littermates, body weight (mean±S.E.M), 24.1 ±1.4g vs
611 24.3±1.3g , respectively) were fed 60% HFD for 2 weeks. Each genotype was then
612 further split into vehicle or metformin (300mg/kg) treatment group, given a single
613 gavage dose at 8am and fasted for 6 hrs. At time of GTT, body weights
614 (mean±S.E.M) of study groups were 26.4.1±1.5g; 26.5±1.0g for wild type Vehicle
615 and Metformin treatment respectively, and 25.6±1.2g; 27.1±1.3g for Gdf15^{-/-}
616 Vehicle and Metformin treatment respectively (1 way ANOVA, p=0.8722). Baseline
617 testing as mouse study 6. Mice then received a single dose of 20% glucose via
618 intraperitoneal route (2mg/g dose) with serial measurement of glucose levels
619 measured at time points indicated. Sacrifice and insulin analysis as mouse study 6.

620

621 **Mouse study 8. Acute single high dose metformin study in high fat diet fed**
622 **wild type mice.**

623 Male C57BL6/J mice (Charles River, Margate, UK) aged 14 weeks were switched
624 from standard chow to 45 %HFD fat (D12451i) for 1 week then 60%HFD (D12492i,
625 for 3 weeks). At time of study (18 weeks old) body weights (mean ±SEM) were 40.4±
626 1.2g vs 41.1±1.3g, vehicle vs metformin group, respectively. 500mg of metformin
627 (Sigma, PHR1084-500MG) was dissolved in 8.35 mls of water to make a working
628 stock of 60mg/ml. Mice received a single dose by oral gavage of either 600mg/kg

629 metformin or matched volume of vehicle (water). They were returned to ad lib 60 %
630 fat diet and 6 hrs later blood was collected as study 2. Tissue samples for RNA
631 analysis were collected into Lysing Matrix D homogenisation tube (MP Biomedicals)
632 on dry ice and stored at -80°C until processed. Intestine between pylorus of stomach
633 and caecum was laid out into 3 equal parts, with tissue taken from mid-point of each
634 third labelled as “proximal”, “ middle” and “ distal” (adapted from ³⁴). Colon section
635 was from mid-point between caecum and anus. Tissue for in-situ hybridisation were
636 dissected and placed into 10% formalin/PBS for 24hr at room temp, transferred to
637 70% ethanol, and processed into paraffin. 5µm sections were cut and mounted onto
638 Superfrost Plus (Thermo-Fisher Scientific). Detection of Mouse Gdf15 was
639 performed on FFPE sections using Advanced Cell Diagnostics (ACD) RNAscope®
640 2.5 LS Reagent Kit-RED (Cat No. 322150) and RNAscope® LS 2.5 Probe Mm-
641 Gdf15-O1 (Cat No. 442948) (ACD, Hayward, CA, USA). Briefly, sections were baked
642 for 1 hour at 60°C before loading onto a Bond RX instrument (Leica Biosystems).
643 Slides were deparaffinized and rehydrated on board before pre-treatments using
644 Epitope Retrieval Solution 2 (Cat No. AR9640, Leica Biosystems) at 95°C for 15
645 minutes, and ACD Enzyme from the LS Reagent kit at 40°C for 15 minutes. Probe
646 hybridisation and signal amplification was performed according to manufacturer’s
647 instructions. Fast red detection of mouse Gdf15 was performed on the Bond RX
648 using the Bond Polymer Refine Red Detection Kit (Leica Biosystems, Cat No.
649 DS9390) according to the ACD protocol. Slides were then counterstained with
650 haematoxylin, removed from the Bond RX and were heated at 60°C for 1 hour,
651 dipped in Xylene and mounted using EcoMount Mounting Medium (Biocare Medical,
652 CA, USA. Cat No. EM897L).

653 Slides imaged on an automated slide scanning microscope (Axioscan Z1 and
654 Hamamatsu orca flash 4.0 V3 camera) using a 20x objective with a numerical
655 aperture of 0.8. Hybridisation specificity was confirmed by the absence of staining in
656 *Gdf15*^{-/-} mice.

657 RNA extraction was carried out with approximately 100mg of tissue in 1ml Qiazol
658 Lysis Reagent (Qiagen 793061) using Lysing Matrix D homogenisation tube and
659 Fastprep 24 Homogeniser (MP Biomedicals) and Qiagen RNeasy Mini kit (Cat no
660 74106) with DNase1 treatment following manufacturers' protocols. 500ng of RNA
661 was used to generate cDNA using Promega M-MLV reverse transcriptase followed
662 by TaqMan qPCR in triplicates for GDF15. Samples were normalised to Act B.
663 TaqMan Probes: Mm00442228 m1 GDF15, Mm02619580_g1 Act B, TaqMan;2X
664 universal PCR Master mix (Applied Biosystems Thermo Fisher 4318157);
665 QuantStudio 7 Flex Real time PCR system (Applied Biosystems Life Technologies)

666 **Mouse study 9. Acute phenformin study in standard chow-fed wild type**
667 **animals.**

668 Male C57BL6/J mice aged 14 weeks with supplier, protocol and methods as study 2,
669 except phenformin (Sigma PHR1573-500mg) used instead of metformin.

670 **Organoid studies.**

671 Duodenal and ileal mouse organoid line generation, maintenance and 2D culture
672 was performed as previously described³⁵. CHOP null mice were kind gift of Dr Jane
673 Goodall (University of Cambridge), with line from Jackson Laboratory, Maine
674 (B6.129S(Cg)-Ddit3tm2.1Dron/J, Stock No: 005530) Human rectal organoids
675 (experiments approved by the Research Ethics Committee under license number
676 09/H0308/24) were generated from fresh surgical specimens (Tissue Bank

677 Addenbrooke's Hospital (Cambridge, UK)) following a modified protocol^{35,36}. Briefly
678 rectal tissue was chopped into 5mm fragments and incubated in 30 mM EDTA for
679 3x10mins, with tissue shaken in PBS after each EDTA treatment to release intestinal
680 crypts. The isolated crypts were then further digested using TrypLE (Life
681 Technologies) for 5 mins at 37⁰C to generate small cell clusters. These were then
682 seeded into basement membrane extract (BME, R&D technology), with 20 µl domes
683 polymerised in multiwell (48) dishes for 30-60 mins at 37⁰C. Organoid medium (Sato
684 et al 2011) was then overlaid and changed 3 times per week. Human organoids were
685 passaged every 14-21 days using TrypLE digestion for 15 mins at 37⁰C, followed by
686 mechanical shearing with rigorous pipetting to breakup organoids into small clusters
687 which were then seeded as before in BME. For transwell experiments TrypLE
688 digested organoids were seeded onto matrigel (Corning) coated (2% for 60 mins at
689 37⁰C) polyethylene Terephthalate cell culture inserts, pore size 0.3 µm (Falcon) in
690 organoid medium supplemented with Y-27632 (R&D technology). Organoids were
691 observed through the transparent cell inserts to ensure 2D culture formation
692 (allowing apical cell access for drug treatments). Medium was changed after 2 days
693 and then switched on day 3 to a differentiation medium with wnt3A conditioned
694 medium reduced to 10% and SB202190 / nicotinamide omitted from culture for 5
695 days.

696 For GDF 15 secretion experiments 2D cultured organoid cells were treated for 24 hrs
697 with indicated drugs, with medium then collected and GDF15 measured at the Core
698 Biochemical Assay Laboratory (Cambridge) using the human or mouse GDF15
699 assay kit as outlined in CAMERA human study and mouse study 2 above.

700 RNA was extracted using TRI reagent (Sigma), with any contaminated DNA
701 eliminated using DNA free removal kit (Invitrogen). Purified RNA was then reverse

702 transcribed using superscript II (Invitrogen) as per manufacturer's protocol. RT-
703 qPCR was performed on a QuantStudio 7 (Applied Biosystems) using Fast Taqman
704 mastermix and the following probes (Applied Biosystems); Human GDF15
705 (Hs00171132_m1), Human ACTB (Hs01060665_g1). Gene expression was
706 measured relative to β -actin in the same sample using the Δ Ct method, with fold (cf.
707 control) shown for each experiment.

708 **Hepatocyte studies.**

709 **Primary mouse hepatocyte isolation and culture.**

710 Hepatocytes from 8-12 week old C57B6J male mice were isolated by retrograde,
711 non-recirculating in situ collagenase liver perfusion. In brief: livers were perfused with
712 modified Hanks medium without calcium (NaCl- 8.0 g/L; KCl- 0.4 g/L; MgSO₄.7H₂O-
713 0.2 g/L; Na₂HPO₄.2H₂O- 0.12 g/L; KH₂PO₄- 0.12 g/L; Hepes- 3 g/L; EGTA- 0.342
714 g/L; BSA- 0.05 g/L) followed by digestion with perfusion media supplemented with
715 calcium (CaCl₂.2H₂O- 0.585 g/L) and 0.5mg/ml of collagenase IV (Sigma, C5138).
716 The digested liver was removed and washed using chilled DMEM:F12 (Sigma)
717 medium containing 2 mM L-glutamine, 10 % FBS, 1% penicillin/streptomycin
718 (Invitrogen). Viable cells were harvested by Percoll (Sigma) gradient. The final pellet
719 was resuspended in the same DMEM:F12 media. Cell viability was greater than
720 90%. Hepatocytes were plated onto primaria plates (Corning). Hepatocytes were
721 allowed to recover and attach for 4-6 hr before replacement of the medium overnight
722 prior to stress treatments the following day for the times and concentrations
723 indicated.

724 **Generation and culture of iPSC derived human hepatocytes.**

725 The human induced pluripotent cell (hiPSC) line A1ATDR/R used in this work was
726 derived as previously described ^{37,38} under approval by the regional research ethics
727 committee (reference number 08/H0311/201). hiPSCs were maintained in Essential
728 8 chemically defined media³⁹ supplemented with 2ng/ml Tgf- β (R&D) and 25ng/ml
729 FGF2 (R&D), and cultured on plates coated with 10 μ g/ml Vitronectin XFTM
730 (STEMCELL Technologies). Colonies were regularly passaged by short-term
731 incubation with 0.5mM EDTA in PBS. For hepatocyte differentiation, colonies were
732 dissociated into single cells following incubation with StemPro™ Accutase™ Cell
733 Dissociation Reagent (Gibco) for 5 minutes at 37°C. Single cell suspensions were
734 seeded on plates coated with 10 μ g/ml Vitronectin XFTM (STEMCELL Technologies)
735 in maintenance media supplemented with 10 μ M ROCK Inhibitor Y-27632
736 (Selleckchem) and grown for up to 72h prior to differentiation. Hepatocytes were
737 differentiated as previously reported⁴⁰, with minor modifications as listed. Briefly,
738 following endoderm differentiation, anterior foregut specification was achieved after 5
739 days of culture with RPMI-B27 differentiation media supplemented with 50ng/ml
740 Activin A (R&D)⁴⁰. Foregut cells were further differentiated into hepatocytes with
741 HepatoZYME-SFM (Gibco) supplemented with 2mM L-glutamine (Gibco), 1%
742 penicillin-streptomycin (Gibco), 2% non-essential amino acids (Gibco), 2%
743 chemically defined lipids (Gibco), 14 μ g/ml of insulin (Roche), 30 μ g/ml of transferrin
744 (Roche), 50 ng/ml hepatocyte growth factor (R&D), and 20 ng/ml oncostatin M
745 (R&D), for up to 27 days.

746

747 **Cellular studies on integrated stress response.**

748 **Chemicals and Reagents.**

749 Tunicamycin and ISRIB were purchased from Sigma-Aldrich. Metformin and
750 Phenformin was purchased from Cayman Chemicals and GSK2606414 from
751 Calbiochem. The antibody for GDF15 and CHOP (sc-7351) were obtained from
752 Santa Cruz. Phospho S51 EIF2 α (ab32157) and Calnexin (ab75801) were
753 purchased from Abcam. The antibody for ATF4 was a kind gift from Dr David Ron
754 (CIMR, Cambridge).

755 **Eukaryotic cell lines and treatments.**

756 Mouse embryonic fibroblast (MEF) cells lines were obtained from David Ron
757 (CIMR/IMS, Cambridge) and maintained as previously described¹⁸. MEFs were
758 transfected with 30 nM control siRNA or a smartpool on-target plus siRNA for mouse
759 CHOP (Dharmacon - L-062068-00-0005) using Lipofectamine RNAi MAX
760 (Invitrogen) according to the manufacturer's instruction. 48 h post siRNA
761 transfection, cells were processed for RNA and protein expression analysis. All cells
762 were maintained at 37 °C in a humidified atmosphere of 5 % CO₂ and seeded onto
763 6- or 12-well plates prior to stress treatments for the times and concentrations
764 indicated. Vehicle treatments (e.g. DMSO) were used for control cells when
765 appropriate.

766 **RNA isolation/cDNA synthesis/Q-PCR.**

767 Following treatments, cells were lysed with Buffer RLT (Qiagen) containing 1 % 2-
768 Mercaptoethanol and processed through a Qiashreder with total RNA extracted
769 using the RNeasy isolation kit according to manufacturer's instructions (Qiagen).
770 RNA concentration and quality was determined by Nanodrop. 400 ng - 500 ng of
771 total RNA was treated with DNase1 (Thermofisher Scientific) and then converted to
772 cDNA using MMLV Reverse Transcriptase with random primers (Promega).

773 Quantitative RT-PCR was carried out with either TaqMan™ Universal PCR Master
774 Mix or SYBR Green PCR master mix on the QuantStudio 7 Flex Real time PCR
775 system (Applied Biosystems). All reactions were carried out in either duplicate or
776 triplicate and Ct values were obtained. Relative differences in the gene expression
777 were normalized to expression levels of housekeeping genes, HPRT or GAPDH for
778 cell analysis, using the standard curve method. Primers used for this study: mouse
779 GDF15 (Mm00442228_m1 – ThermoFisher Scientific), human GDF15
780 (Hs00171132_m1 - ThermoFisher Scientific), human GAPDH (Hs02758991_g1 –
781 ThermoFisher Scientific), mouse HPRT (Forward – AGCCTAAGATGAGCGCAAGT,
782 reverse - GGCCACAGGACTAGAACACC)

783 **Immunoblotting.**

784 Following treatments, cells were washed twice with ice cold D-PBS and proteins
785 harvested using RIPA buffer supplemented with cOmplete protease and PhosStop
786 inhibitors (Sigma). The lysates were cleared by centrifugation at 13 000 rpm for 15
787 min at 4 °C, and protein concentration determined by a Bio-Rad DC protein assay.
788 Typically, 20-30 µg of protein lysates were denatured in NuPAGE 4x LDS sample
789 buffer and resolved on NuPage 4-12 % Bis-Tris gels (Invitrogen) and the proteins
790 transferred by iBlot (Invitrogen) onto nitrocellulose membranes. The membranes
791 were blocked with 5 % nonfat dry milk or 5 % BSA (Sigma) for 1 h at room
792 temperature and incubated with the antibodies described in the reagents section.
793 Following a 16 h incubation at 4 °C, all membranes were washed five times in Tris-
794 buffered saline-0.1% Tween-20 prior to incubation with horseradish peroxidase
795 (HRP)-conjugated anti-rabbit immunoglobulin G (IgG), HRP-conjugated anti-mouse
796 IgG (Cell Signalling Technologies). The bands were visualized using Immobilon

797 Western Chemiluminescent HRP Substrate (Millipore). All images were acquired on
798 the ImageQuant LAS 4000 (GE Healthcare).

799 **Statistical analyses.**

800 CAMERA data were analysed using a mixed linear model with restricted maximum
801 likelihood to investigate the metformin effect on GDF-15. This is analogous to
802 conducting a repeated measures ANOVA, but is a more flexible analysis and allows
803 for missing observations within subject. The 0-18 months difference in weight and
804 GDF15 correlation was tested using Spearman's coefficient. CAMERA data were
805 analysed using STATA version 15.1.

806 Other statistical analyses were performed using Prism 7 and Prism 8, using
807 unpaired 2 tailed t-tests , or 2-way ANOVA, with multiple comparison adjustment by
808 Tukey's or Sidak's test. Metabolic rate was determined using ANCOVA with energy
809 expenditure as the dependent variable, body weight as a covariate and treatment as
810 a fixed factor. ANCOVA and analyses of glucose and insulin tolerance testing in
811 mice were performed using SPSS 25 (IBM).

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814 **Data availability.**

815 The data that support the findings of this study are available from the corresponding
816 authors upon request. The CAMERA trial dataset is held at the University of
817 Glasgow and is available on request from the investigators subject to a signed
818 agreement operating within the confines of the original ethics application.

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888

889 **Author Contributions.**

890

891 Overall conceptualization of studies included in this body of work by A.P.C., N.S.,
892 D.B.S., B.B.A. and S.O'R. These authors contributed equally to this work.

893 A.P.C., M.C., P.T., D.Rimmington, I.C. and Y.C.L.T. designed, managed, performed

894 and analysed data from mouse experiments. S.V. designed experiments and

895 analysed data. A.M. and G.S.H.Y. contributed to conceptualisation of experiments

896 and data analysis. J.T. performed ISH experiments. S.P. designed, managed and

897 performed cell based assays along with E.L.M., S.R.C., R.A.T., H.P.H., A.V-P., L.V.

898 and D.Ron. J.T.J.H. undertook measurement of serum metformin levels .M.Y.,

899 D.A.G., F.M.G., F.R. designed, performed and analysed organoid experiments.

900 A.R.K., R.R.E. and K.S.N. designed and performed short term metformin studies in

901 humans. N.J.W undertook analysis of Ely Study Cohort. P.W., D.P. and N.S.

902 designed, analysed and interpreted data arising from the CAMERA study. A.P.C.,

903 D.B.S., B.B.A. and S.O'R. wrote the paper, which was reviewed and edited by all the

904 authors.

905

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916 **Materials and correspondence.**

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922 **Extended Data Figures Legends.**

923 **Extended Data Figure 1. Expanded CAMERA data set.**

924 **a**, Linear association between change in body weight and change in plasma GDF15
925 between 0 and 18 months among metformin treated participants (n=74, Spearman
926 correlation $r=-0.26$, two-sided $p=0.024$). Red line is linear regression slope, and grey
927 area is 95% confidence interval for slope.

928 **b**, Absolute and relative differences in plasma GDF15 concentration between
929 metformin and placebo groups at each time point (total 625 observations in 173
930 participants).

931 **c,d**, Individual measures of plasma GDF15 levels in placebo group (c) and
932 metformin group (d) over time.

933 **e**, Plasma GDF15 concentration (95%CI) in overweight or obese non-diabetic
934 participants with known cardiovascular disease randomised to metformin or placebo
935 in CAMERA; modelled using a mixed linear model as per Figure 1 and grouped as
936 "all participants" and "all participants not reporting diarrhoea and vomiting". Model
937 includes all participants

938

939 **Extended Data Figure 2. Effect of single oral dose of metformin in chow fed** 940 **mice.**

941 Serum GDF15 levels in male mice measured 2, 4, or 8 hours after a single gavage
942 dose of metformin (300mg/kg). **a**, mice *ad libitum* overnight fed prior to gavage. **b**,

943 mice fasted for 12 hour prior to gavage. Data are mean \pm SEM (**a**; n=6/group, **b**; n=
944 4/group); P by 2-way ANOVA with Tukeys correction for multiple comparisons.

945

946 **Extended Data Figure 3. Body weight changes with metformin treatment in**
947 **mice with disrupted GDF15-GFRAL signalling.**

948 **a**, Absolute body weight in *Gdf15*^{+/+} and *Gdf15*^{-/-} mice on a high-fat diet treated with
949 metformin (300mg/kg/day) for 11 days, mice as **Figure 2a**. Data are mean \pm SEM, P
950 by 2-way ANOVA with Tukey's correction for multiple comparisons.

951 **b**, Absolute body weight in high fat diet fed *Gfral*^{+/+} and *Gfral*^{-/-} mice given oral
952 dose of metformin (300mg/kg) once daily for 11 days, mice as **Figure 2c**. Data are
953 mean \pm SEM.

954 **c**, Absolute body weight of metformin-treated, obese mice dosed with an anti-GFRAL
955 antagonist antibody or with control IgG weekly for 5 weeks starting 4 weeks after
956 initial metformin exposure, mice as **Figure 2d**. Data are mean \pm SEM. P by 2-way
957 ANOVA with Tukey's correction for multiple comparisons.

958

959 **Extended Data Figure 4. Response of high fat diet fed *Gdf15*^{-/-} and *Gfral*^{-/-} mice**
960 **to metformin.**

961 **a**, Circulating GDF15 levels in high fat diet fed *Gdf15*^{+/+} and *Gdf15*^{-/-} mice given
962 oral dose of metformin (300mg/kg) once daily for 11 days. Data are mean \pm SEM,
963 mice as **Figure 2a**. All samples from *Gdf15*^{-/-} were below lower limit of assay (<
964 2pg/ml), P value by 2-way ANOVA with Tukey's correction for multiple comparisons.

965 **b**, Circulating GDF15 levels in high fat diet fed *Gfral*^{+/+} and *Gfral*^{-/-} mice given oral
966 dose of metformin (300mg/kg) once daily for 11 days. Data are mean \pm SEM, mice
967 as **Figure 2c**, P by 2-way ANOVA with Tukey's correction for multiple comparisons.

968 **c**, Cumulative food intake in high fat diet fed *Gfral*^{+/+} and *Gfral*^{-/-} mice on a high fat
969 diet given oral dose of metformin (300mg/kg) once daily for 11 days. Data are mean
970 \pm SEM, mice as **Figure 2c**, non-significant difference vehicle vs metformin by 2W
971 ANOVA.

972 **d**, Fat mass (left panel) and lean mass (right panel) in metformin-treated obese
973 mice dosed with an anti-GFRAL antagonist antibody, weekly for 5 weeks, starting 4
974 weeks after initial metformin exposure (mice as **Figure 2d**). Body composition was
975 measured using MRI after 4 weeks of metformin exposure, prior to receiving anti-
976 GFRAL (week 4), after 6 weeks of metformin exposure and 2 weeks after receiving
977 anti-GFRAL (week 6) and after 9 weeks of metformin exposure and 5 weeks after
978 receiving anti-GFRAL (week 9). Data are mean \pm SEM (n=7 Vehicle + control IgG
979 and Metformin + anti - GFRAL; n=8 other groups); P by 2-way ANOVA with Tukey's
980 correction for multiple comparisons.

981

982 **Extended Data Figure 5. Response of second, independent cohort of high-fat**
983 **diet fed *Gdf15*^{+/+} and *Gdf15*^{-/-} mice to metformin.**

984 **a,b,c**, Percentage change in body weight (**a**), absolute body weight (**b**) and
985 cumulative food intake (**c**) in *Gdf15*^{+/+} and *Gdf15*^{-/-} mice on a high-fat diet treated
986 with metformin (300mg/kg/day) for 11 days. Data are mean ± SEM (n=6/group,
987 except *Gdf15*^{-/-} vehicle= 7), P by 2-way ANOVA with Tukey's correction for multiple
988 comparisons.

989 **d**, Circulating metformin levels in mice 6 hrs after final dose of metformin on day 11.
990 Data are mean ± SEM (n=6/group, except *Gdf15*^{+/+} vehicle= 4, *Gdf15*^{-/-} vehicle=
991 7), P by 2-way ANOVA with Tukey's correction for multiple comparisons.

992 **Extended Data Figure 6. Glucose, insulin and GDF15 response to metformin.**

993 **a**, Fasting glucose from OGTT as **Figure 3e** and **3f**. ANOVA analysis, effect of
994 antibody p= 0.028, effect of metformin p= 0.271, interaction of antibody and
995 metformin p 0.707.

996 **b**, Circulating GDF15 in mice undergoing ipGTT post single dose metformin as
997 **Figure 3 k** and **3l**. P by 2-way ANOVA with Tukey's correction for multiple
998 comparisons.

999 **c,d**, Fasting glucose (**c**) and fasting insulin (**d**) at time 0 of ipGTT as **Figure 3 k** and
1000 **3l**, non-significant by 2-way ANOVA.

1001 **e**, AUC analysis of glucose levels as in **Figure 3k** and **l**. P by 2-way ANOVA, effect of
1002 genotype p= 0.392, interaction of genotype and metformin p= 0.883.

1003 **f**, Circulating GDF15 levels in high-fat diet fed *Gdf15*^{+/+} mice after single oral dose
1004 of metformin (600mg/kg). Samples were collected 6 hours after dosing, data are
1005 mean ± SEM, (n=7/group), P value (95% confidence interval) by two tailed t-test.
1006

1007 **Extended Data Figure 7. a**, Representative images from the mouse with circulating
1008 GDF15 level closest to group median shown in **Fig4b** with images from other regions
1009 of the gut and from liver. **b**, In situ hybridization for *Gdf15* mRNA expression (red
1010 spots) in colon. Tissue collected from high-fat fed wild type mice, 6 hrs after single
1011 dose of oral metformin (600mg/kg)(right side, red box, m1-m7) or vehicle gavage (
1012 left side, blue box, v1-v7), n=7/group, mice as **Figure 4**.

1013 **Extended Data Figure 8. Analysis of *Gdf15* mRNA expression (normalised to**
1014 **expression levels of *ActB*) in tissue from high fat diet fed *Gdf15*^{+/+} mice.**

1015 Metformin dose (300mg/kg) once daily for 11 days (see **Figure 2a**). Data are mean
1016 ± SEM, n=6 metformin, n=7 vehicle, P value (95% confidence interval) by two tailed
1017 t-test.

1018 **Extended Data Figure 9. Hepatic GDF15 response to biguanides.**

1019 **a,b**, *Gdf15* mRNA expression in (**a**) primary mouse hepatocytes or (**b**) human iPSC
1020 derived hepatocytes treated with vehicle control (Con) or metformin for 6 h. mRNA
1021 expression is presented as fold expression relative to control treatment (set at 1),
1022 normalised to *Hprt* and *GAPDH* gene in mouse and human cells, respectively. Data
1023 are expressed as mean ± SEM from four (**a**) and two (**b**) independent experiments. P

1024 value (95% confidence interval) by 1 way ANOVA with Tukey's correction for
1025 multiple comparisons.

1026 **c,d**, Circulating levels of GDF15 (**c**) and hepatic *Gdf15* mRNA expression (**d**)
1027 (normalised to $\beta 2$ microglobulin) in chow fed, wild type mice 4 hrs after single oral
1028 dose of phenformin (300mg/kg). Data are mean \pm SEM, n= 6/group, P value (95%
1029 confidence interval) by two tailed t-test.

1030 **e**, Representative image of in situ hybridization for *Gdf15* mRNA expression (red
1031 spots) of fixed liver tissue derived from animals treated as described in (**c**) and (**d**).

1032 **Extended 10. Role of the Integrated Stress Response (ISR) in biguanide-** 1033 **induced Gdf15 expression**

1034 **a,b**, mRNA levels in kidney (**a**) and colon (**b**) isolated from obese mice 24 hours after
1035 a single oral dose of metformin (600mg/kg). Data are mean \pm SEM (n=5/group). P
1036 values (95% confidence interval) by two tailed t-test. *Gdf15* mRNA fold induction 24
1037 hrs post metformin 600mgs/kg is positively correlated with CHOP mRNA induction in
1038 both kidney (**a**, right panel) and colon (**b**, right panel), black line= linear regression
1039 analysis.

1040 **c-g**, Immunoblot analysis of ISR components (**c**) and *Gdf* mRNA expression (**d**) in
1041 wild type MEFs (mouse embryonic fibroblasts) treated with vehicle control (Con),
1042 metformin (Met, 2 mM) or phenformin (Phen, 5 mM) or tunicamycin (Tn, 5 μ g/ml -
1043 used as a positive control) for 6 hrs. **e**, *Gdf15* mRNA expression in ATF4 knockout
1044 (KO) MEFs or (**f**) in control siRNA and CHOP siRNA transfected wild type MEFs
1045 treated with Tn or Phen for 6 hrs or (**g**) in wild type MEFs pre-treated for 1 h either
1046 with the PERK inhibitor GSK2606414 (GSK, 200 nM) or eIF2 α inhibitor ISRIB (ISR,
1047 100 nM) then co-treated with Phen for a further 6 hrs. mRNA expression is presented
1048 as fold-expression relative to its respective control treatment (set at 1) or phen
1049 treated samples (set as 100) with normalisation to *Hprt* gene expression. Data are
1050 expressed as mean \pm SEM from two for (**c**) and (**d**) and at least three independent
1051 experiments for (**e-g**). P value (95% confidence interval) by two tailed t-test relative
1052 to Phen treated control wild and control siRNA treated samples.

1053 **h**, GDF15 protein in supernatant of mouse derived 2D duodenal organoids treated
1054 with metformin in the absence or presence of ISRIB (1 μ M). Data are expressed as
1055 mean \pm SEM from two independent experiments. From each well, measurement of
1056 protein was at least in duplicate. P by 2 way ANOVA with Sidak's correction for
1057 multiple comparisons.

1058 **i**, GDF15 protein in supernatants of mouse-derived 2D duodenal organoids from wild
1059 type and CHOP null mice treated with metformin from two independent experiments
1060 From each well, measurement of protein was at least in duplicate. Data are mean \pm
1061 SEM, P value (95% confidence interval) by two-tailed t-test.

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