

SUPPLEMENTARY MATERIALS AND METHODS

Liver fat measurements

Proton magnetic resonance spectroscopy was used for the quantification of intra-hepatic fat content. Briefly, three 20 x 20 x 20-mm³ spectroscopic volumes of interest were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margins of the liver. Liver fat percentage was then calculated as the mean value of the liver fat percentages in the three aforementioned liver segments.

Assessment of hepatic mitochondrial function (13C-ketoisocaproate breath test)

The 13C-ketoisocaproate breath test (13C-KICA BT) protocol was undertaken in patients with NAFLD as previously described (1). Subjects refrained from alcohol and had fasted overnight for at least 12 h prior to each test. All subjects were at rest for the duration of the study and remained fasted throughout. On the morning of the study, to standardize CO₂ production, subjects were asked to lie down on a bed and carbon dioxide produced (VCO₂) at rest was measured by indirect calorimetry (GEM Nutrition, UK) for 25 min prior to the start of the 13C-KICA BT. Paired breath samples for baseline measurement of isotopic abundance were collected from each subject exhaling directly into 12-ml Exetainer breath tubes (LABCO Ltd, High Wycombe, UK) via straws. Each subject then ingested a solution containing 1 mg kg⁻¹ body weight of 2-keto-[1-¹³C]-isocaproic acid (99% ⁻¹³C; Cambridge Isotopes, USA) along with 20 mg kg⁻¹ body weight of L-leucine (Sigma) in 200 ml of water. Further paired breath samples were then collected every 10 min for 60 min. The enrichment of ¹³C (atom % excess) in CO₂ in breath at time (t) was calculated from the ¹³C abundance measurements by continuous flow isotope ratio mass spectrometry (CF-IRMS, ABCA System, SERCON, Crewe, UK) and used with a measure of the total CO₂ production to calculate the cumulative percent ¹³C-dose recovered over 1 h (cPDR over 1 h). The cPDR over 1 h has been previously validated as a measure of HMF (2-4).

Liver fibrosis biomarkers

Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA) have been analysed using ELISA Kits (R&D) read on Luminex 200 (Bio-plex 200 - Bio-Rad, Watford, UK). The Procollagen-III N-terminal Propeptide (PIIINP) assay has been performed with a UniQ radioimmunoassay kit supplied by Orion Diagnostica (Product no.68570). Platelet counts were obtained using a Sysmex XN10 analyser and impedance platelet count. We have generated three different liver fibrosis scores, namely: ELF (5), APRI (6) and FIB-4 (7, 8). The first of these scores has been calculated using measurement of HA, PIIINP and TIMP-1 (5). APRI scores were calculated as previously described with an upper limit of normal for AST of 40 IU/L (6). FIB-4 scores were obtained as previously described (7, 8).

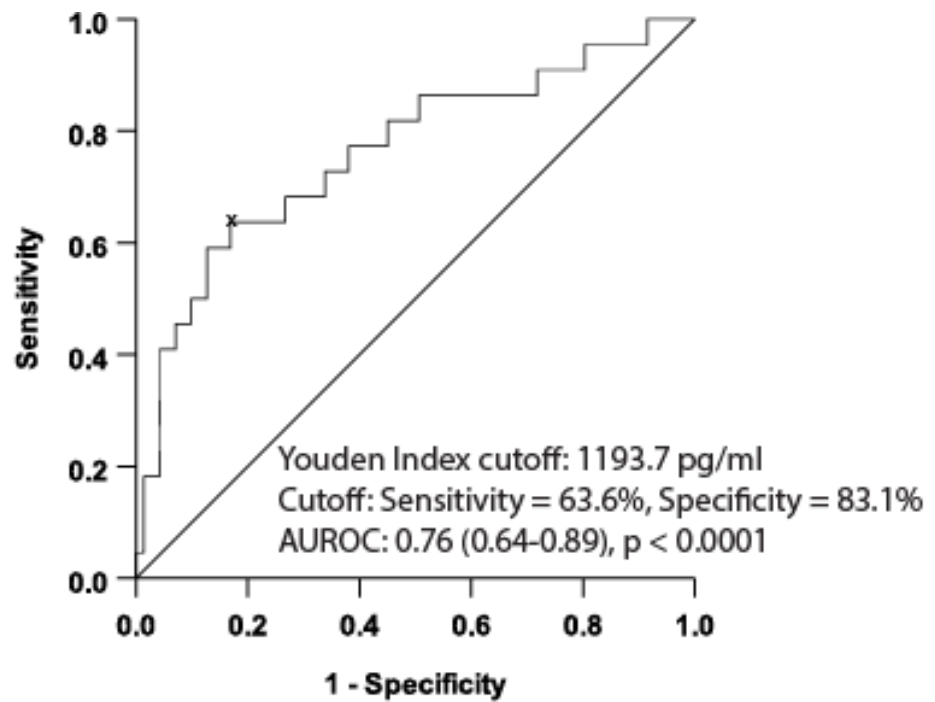
Appetite, hunger and satiety assessment

Participants were instructed to abstain from alcohol consumption and strenuous physical activity for 2 days prior to the day of the satiety test (9, 10). Participants were offered a free-choice buffet breakfast (comprising yoghurt, bread, butter, cheese, jam, fruit, orange juice and water; approximately 470 kcal (11)) and were instructed to complete this within 15 minutes. Food and drink were weighed before and after the meal and energy intake was calculated accordingly. Before and after breakfast (at -5, 0, 15, 30, 60, 120 and 180 minutes), appetite ratings were assessed using a 100-mm visual analogue scale (12). Venous blood samples were also taken at each time-point and used to measure plasma satiety hormone concentrations (see Methods section in manuscript text. For the assessment of appetite, a satiety visual analogue scale with verbal descriptors expressing the most positive and the most negative ratings positioned at each end of a 100-mm line. Participants were asked to draw a vertical mark across the line corresponding to their feelings from 0 (not hungry at all) to 100 (very hungry). Satiety was measured by the distance from the left end of the line to the mark placed by each participant (12). The area under the curve (AUC) values were calculated using the trapezoidal rule for both participants reported satiety and satiety hormone concentrations and are expressed as (mm/min) and (pmol/mol/min), respectively.

Gut microbiota analyses – DNA extraction, sequencing, bioinformatics, and statistical analysis

Initial quality-filtering of the reads was conducted with the Illumina Software, yielding an average of 169,858 pass-filter reads per sample. with the FastQC software (<http://www.bioinformatics.babraham.ac.uk/publications.html>), and reads were trimmed to 220 bp (R1) and 200 bp (R2) with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The reads were merged with the merge-Illumina-pairs application (with P = 0.03, enforced Q30 check, perfect matching to primers, which are removed by the software, and otherwise default settings including no ambiguous nucleotides allowed) (13). For all samples but two, a subset of 26,000 reads was randomly selected using Mothur (14) to avoid large disparities in the number of sequences. Subsequently, the UPARSE pipeline implemented in USEARCH (15) was used to further process the sequences. Amplicon sequence variants (ASVs) were identified using UNOISE3 (16). Taxonomic prediction was performed using the *nbc_tax* function (17), an implementation of the RDP Naive Bayesian Classifier algorithm (18). Raw sequences are available in the SRA database (PRJNA559052). For the statistical analysis of the gut microbiota, the bacterial taxa with a proportional count across all samples below 0.01% and/or present in less than 35% of the patients were removed. Bacterial taxa significantly different between tertiles of serum GDF-15 concentrations were identified using the Mann-Whitney U test, with a Benjamini-Hochberg correction for multiple testing – between tertile gut microbiota analyses are presented as a q-value, which is adjusted for the false discovery rate (i.e. a FDR cut-off of $q = 0.05$ was considered to be statistically significant). Between tertile gut microbiota analyses were performed in R statistical software.

SUPPLEMENTARY FIGURE



Supplementary figure 1 – Receiver-operating characteristic curve of serum GDF-15 concentrations for \geq F3 fibrosis (≥ 9.7 kPa as measured by validated VCTE threshold). State variable was VCTE derived kPa measurements < 9.7 kPa vs. ≥ 9.7 kPa (0 and 1 respectively). Sample size $n = 93$.

SUPPLEMENTARY TABLES

Supplementary table 1 – Measures of appetite and satiety stratified by T2DM status.

Variables	Without T2DM (n = 56)	With T2DM (n = 42)	p-value
Hunger (mm)	40.0 ± 23.5	41.7 ± 22.7	0.85
Fullness (mm)	31.0 (38)	21.0 (40.0)	0.41
Mean satiety (mm)	51.7 ± 11.1	51.6 ± 11.9	0.83
Ghrelin (pg/ml)	94.9 (76.8)	60.2 (60.1)	0.001
GIP (pg/ml)	44.2 (34.6)	47.4 (34.1)	0.38
GLP-1 (pg/ml)	151.6 (47.7)	181.4 (95)	0.009
PYY (pg/ml)	110.0 (69.9)	128.6 (91.4)	0.1
PP (pg/ml)	46.9 (63.1)	72.6 (93.6)	0.08
Total calories consumed (kcal)	285.0 (82.0)	256.7 (107.0)	0.08
Hunger (mm/min)	35.8 ± 16.4	40.1 ± 19.5	0.24
Fullness (mm/min)	57.7 (26.1)	53.4 (29.1)	0.06
Mean satiety (mm/min)	61.3 (14.4)	62.6 (10.6)	0.5
AUC Ghrelin (pg/ml/min)	69.3 (66.6)	55.8 (59.4)	0.02
AUC GIP (pg/ml/min)	173.6 (90.4)	177.7 (110.6)	0.59
AUC GLP-1 (pg/ml/min)	161.8 (44.8)	205.5 (85.5)	<0.0001
AUC PYY (pg/ml/min)	105.0 (63.2)	122.0 (83.3)	0.03
AUC PP (pg/ml/min)	146.0 (139.3)	239.3 (270.4)	0.02

Sample size, n=98. Data for satiety hormones was available for: Without T2DM ≥52 and with T2DM ≥38. Data presented as mean ± SD or median (inter-quartile range). Abbreviations: T2DM: type 2 diabetes mellitus, GIP: gastric inhibitory polypeptide, GLP-1: glucagon-like peptide, PYY: peptide YY, PP: pancreatic polypeptide

Supplementary table 2 - Binary logistic regression models showing associations between other liver fibrosis biomarkers and VCTE kPa measurement cut offs for the identification of \geq F2 and \geq F3 liver fibrosis.

Biomarker	Model	OR (95%CI)	P-value
APRI	1a	10.95 (1.85-64.77)	0.008
		11.81 (1.82-76.6) #	0.01
	2a	10.84 (1.78-65.92)	0.01
		11.34 (1.75-73.64) #	0.01
	3a	11.63 (1.9-73.0)	0.009
		11.36 (1.75-73.73) #	0.01
FIB-4	1b	2.73 (1.33-5.59)	0.006
		2.23 (1.09-4.55) #	0.03
	2b	2.84 (1.16-6.94)	0.02
		2.79 (1.1-7.02) #	0.03
	3b	2.94 (1.19-7.3)	0.02
		2.80 (1.11-7.05) #	0.03
ELF	1c	2.88 (0.82-10.10)	0.10
		2.53 (0.65-9.85) #	0.18
	2c	2.32 (0.58-9.33)	0.24
		2.67 (0.59-12.20) #	0.20
	3c	2.37 (0.59-9.60)	0.22
		2.68 (0.59-12.26) #	0.20

Dependent variables were < F2 liver (< 8.2 kPa) vs \geq F2 liver (\geq 8.2 kPa) or < F3 liver (< 9.7 kPa) vs \geq F3 liver (\geq 9.7 kPa) the latter of which is denoted as #. Variables within each model are Model 1a-c; biomarker only, Model 2a-c; biomarker and age and Model 3a-c; biomarker, age and sex. For models including either APRI or FIB-4 sample size, n=94. For models including ELF sample size, n=88.

Abbreviations: APRI: AST to platelet ratio index, FIB-4: Fibrosis-4, ELF: enhanced liver fibrosis

Supplementary table 3 – Multivariable linear regression model showing associations with liver VCTE kPa measurement and other liver fibrosis biomarkers

Outcome variable	Independent variables	Unstandardised Coefficient(s)		
		β	95% CI	p-value
A - Liver VCTE measurement				
	Log GDF-15 (pg/ml)	0.35	0.15-0.56	0.001
	Age (yrs)	-0.002	-0.01-0.001	0.23
	Sex (F vs. M)	0.04	-0.09-0.16	0.54
	Total body fat (%)	0.001	-0.01-0.01	0.91
	T2DM status (yes vs.no)	0.009	-0.08-0.09	0.83
	e-GFR (ml/min/1.73 m ²)	0.003	-0.002-0.01	0.27
	AST (IU/l)	0.0004	-0.001-0.002	0.67
B - ELF				
	Log GDF-15 (pg/ml)	0.50	0.09-0.9	0.017
	Age (yrs)	0.01	0.004-0.02	0.001
	Sex (F vs. M)	-0.07	-0.31-0.17	0.54
	Total body fat (%)	0.003	-0.013-0.02	0.68
	T2DM status (yes vs.no)	-0.05	-0.22-0.12	0.55
	e-GFR (ml/min/1.73 m ²)	0.003	-0.01-0.01	0.57
	AST (IU/l)	0.005	0.001-0.01	0.019
C - FIB-4				
	Log GDF-15 (pg/ml)	0.54	0.32-0.75	<0.0001
	Sex (F vs. M)	-0.01	-0.15-0.14	0.92
	Total body fat (%)	0.002	-0.008-0.01	0.68
	T2DM status (yes vs.no)	-0.06	-0.16-0.04	0.25
	e-GFR (ml/min/1.73 m ²)	-0.003	-0.008-0.002	0.28
D - APRI				
	Log GDF-15 (pg/ml)	0.59	0.31-0.87	<0.0001
	Age (yrs)	-0.004	-0.008-0.001	0.10
	Sex (F vs. M)	-0.11	-0.29-0.07	0.23
	Total body fat (%)	0.01	-0.01-0.02	0.33
	T2DM status (yes vs.no)	-0.05	-0.18-0.08	0.43
	e-GFR (ml/min/1.73 m ²)	-0.002	-0.01-0.01	0.63

A) Sample size, n=93. Logarithmically transformed liver VCTE measurements (expressed as kPa) was included as the outcome variable. R² model = 0.261 (P = 0.001). Stepwise regression analysis also identified that (log) GDF-15 concentration explained 21% of the variance in liver VCTE-derived kPa measurement (N.B. the total variance explained by the above regression model was 26.1%). **B)** Sample size, n=85. ELF scores was included as the outcome variable. R² model = 0.425 (P = <0.0001). Stepwise regression analysis also identified that (log) GDF-15 concentrations explained 30% of the variance in ELF scores. (N.B. the total variance explained by the above regression model was 42.5%). **C)** Sample size, n=88. Logarithmically transformed FIB-4 scores was included as the outcome variable. R² model = 0.345 (P = <0.0001). Stepwise regression analysis also identified that

(log) GDF-15 concentrations explained 31.4% of the variance in FIB-4 scores. (N.B. the total variance explained by the above regression model was 34.5%). **D**) Sample size, n=88. APRI scores was included as the outcome variable. R^2 model = 0.249 (P = 0.001). Stepwise regression analysis also identified that (log) GDF-15 concentrations explained 19.1% of the variance in APRI scores. (N.B. the total variance explained by the above regression model was 24.9%). Note - AST was not included when APRI was the outcome variable and AST and age were not included when FIB-4 was the outcome variable given that these factors are involved in the generation of these liver fibrosis biomarkers. Abbreviations: T2DM: type 2 diabetes mellitus, AST: aspartate aminotransferase, VCTE: vibration-controlled transient elastography, APRI: AST to platelet ratio index, FIB-4: Fibrosis-4, ELF: enhanced liver fibrosis, GDF-15; growth differentiation factor-15 and e-GFR: estimated glomerular filtration rate.

Supplementary table 4 – Univariable associations between serum GDF-15 concentrations and measures of appetite and satiety.

Variables	Correlation coefficient(s)	p- value
Ghrelin (pg/ml) ^a	-0.20	0.052
GIP (pg/ml) ^a	0.16	0.14
GLP-1 (pg/ml) ^a	0.18	0.10
PYY (pmol/mol) ^a	0.1	0.36
PP (pg/ml) ^a	0.16	0.14
Hunger (mm)	-0.15	0.13
Fullness (mm) ^a	-0.10	0.32
Mean satiety (mm)	0.02	0.88
Total calories consumed (kcal)	-0.12	0.26
AUC Ghrelin (pg/ml/min)	-0.18	0.09
AUC GIP (pg/ml/min) ^a	0.05	0.67
AUC GLP-1 (pg/ml/min) ^a	0.31	0.003
AUC PYY (pg/ml/min) ^a	0.09	0.40
AUC PP (pg/ml/min) ^a	0.12	0.25
Hunger (mm/min)	-0.16	0.12
Fullness (mm/min)	0.09	0.40
Mean satiety (mm/min) ^a	-0.02	0.86

Sample size, n= ≥87. ^aSpearman's rank correlation coefficients: Abbreviations: GIP: gastric inhibitory polypeptide, GLP-1: glucagon-like peptide, PYY: peptide YY, PP: pancreatic polypeptide.

Supplementary table 5 – Binary logistic regression analysis showing HbA1c concentrations are an independently associated with increased serum GDF-15 concentration.

Variables	OR (95%CI)	P-value
Model 1		
HbA1c (mmol/mol)	1.07 (1.03-2.0)	<0.0001
Model 2		
HbA1c (mmol/mol)	1.08 (1.01-1.15)	0.018
Age (years)	1.08 (1.0, 1.2)	0.05
Metformin treatment (yes vs. no)	34.2 (3.4-346.7)	0.003
hs-CRP (mg/l)	1.65 (1.2-2.23)	0.001
AST (IU/L)	1.12 (1.05-1.2)	0.001
e-GFR (ml/min/1.73 m ²)	0.87 (0.77-0.98)	0.020

Dependent variable was serum GDF-15 concentration < 1193.7 vs. \geq 1193.7 pg/ml (0 and 1 respectively). This threshold concentration of GDF-15 was identified as the Youden index optimal threshold for predicting \geq F2 fibrosis (see Figure 2). Sample size, n=99

Abbreviations: AST: aspartate aminotransferase, VCTE: vibration-controlled transient elastography, GDF-15; growth differentiation factor-15 and e-GFR: estimated glomerular filtration rate.

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