

Glucagon receptor deficiency causes early-onset hepatic steatosis

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ABSTRACT

In mice, glucagon regulates lipid metabolism by activating receptors in the liver, however, its role in human lipid metabolism is incompletely understood. Here we describe three normal weight individuals from a consanguineous family with early-onset hepatic steatosis and/or cirrhosis. Using exome sequencing, we found they were homozygous for two missense variants in the Glucagon Receptor gene (*GCGR*). In cells, the double *GCGR* mutation reduced cell membrane expression and signaling resulting in an almost complete loss of function. Carriers of pathogenic *GCGR* mutations had substantially elevated circulating glucagon and amino acid levels and increased adiposity. Introducing the double *GCGR* mutation into human induced pluripotent stem-cell derived hepatocytes using CRISPR/Cas9, caused increased lipid accumulation. Our results provide an explanation for increased liver fat seen in clinical trials of *GCGR* antagonists and reduced liver fat in people with obesity and steatotic liver disease treated with *GCGR* agonists.

ARTICLE HIGHLIGHTS

- *In this study, we investigated a consanguineous family in whom normal weight individuals had hepatic steatosis and cirrhosis.*
- *Using whole exome sequencing we found two rare homozygous variants in the Glucagon Receptor (GCGR) gene which co-segregated with the phenotype.*
- *In cells, the GCGR mutations result in a loss of function and increased lipid accumulation.*
- *These results highlight the potential risks associated with GCGR antagonists and the benefits of GCGR agonists, currently in clinical trials.*

Rare genetic disorders can provide insights into disease mechanisms which are challenging to dissect in more common heterogeneous forms of metabolic disease, as exemplified by studies in people with monogenic diabetes (1; 2). Glucagon is synthesised by pancreatic alpha-cells and acts as the primary counter-regulatory hormone to insulin (3). Glucagon's effects on carbohydrate and lipid metabolism are transduced by the Glucagon Receptor (GCGR), which is abundantly expressed on hepatocytes. Rare homozygous variants in the Glucagon Receptor gene (*GCGR*) have been reported in patients with pancreatic alpha-cell hyperplasia/neoplasia (4; 5) and in a child with elevated serum amino acid concentrations (6). Here we identified three individuals from a consanguineous family harbouring a homozygous double mutation in *GCGR* associated with early-onset steatotic liver disease despite being normal weight. We performed molecular and clinical studies to interrogate the functional and physiological consequences of almost complete GCGR deficiency in humans.

CASE REPORT

Case 1

The proband (Case 1) was born to UK parents of Pakistani origin who were first cousins (Figure 1A). At age 8 years, he presented with severe meal-related abdominal pain with diarrhoea. He had raised serum alanine aminotransferase levels and an echogenic liver on sonographic examination indicated fatty infiltration, despite being normal weight (Body Mass Index (BMI) 17.3 kg/m², BMI standard deviation score 0.9); there were no clinical features of lipodystrophy. Liver histology showed moderate macrovesicular steatosis, with no inflammation or fibrosis and preserved vascular architecture (Supplementary Figure S1). He had elevated levels of multiple amino acids although the profile was not diagnostic of a known inborn error of metabolism (Table 1). Known causes of hepatitis were excluded (Table 2).

Case 2

The second case (Case 2) was first cousin to both of the proband's parents, and a double first cousin to the proband's mother (Figure 1A). At age 27 years, investigations revealed elevated liver transaminases (alanine aminotransferase 259 IU/L, range 10–49 U/L; aspartate aminotransferase 138 IU/L, range ≤ 34 U/L) and total cholesterol (6.7 mmol/L, range < 5 mmol/L). He did not drink alcohol, did not have type 2 diabetes and had a normal body mass index (BMI 24.6 kg/m²); viral hepatitis was excluded. Liver histology showed macrovascular steatosis with degenerate hepatocytes and clusters of inflammatory cells, consistent with steatohepatitis (Figure 1B). He remained well until he developed type 2 diabetes at age 45 years (BMI 26.3 kg/m²), achieving good glycaemic control with metformin. Cirrhosis was confirmed on Magnetic Resonance Imaging (MRI) which also revealed a 15 mm nodule in the head of the pancreas and pancreatomegaly (Figure 1C). Two years later he presented acutely with confusion, variceal haemorrhage and decompensated cirrhosis. He subsequently underwent liver transplantation. Histological examination of the explanted liver showed cirrhosis with macrovesicular steatosis, ballooning degeneration and Mallory-Denk bodies (Figure 1D). Post-transplantation, he had complete resolution of symptoms related to portal hypertension; the imaging finding of pancreatomegaly had resolved and the pancreatic lesion also reduced in size (MRI: 15 mm to 7 mm).

RESEARCH DESIGN AND METHODS

Study patients and ethical approval

All studies were approved by UK Local Research Ethics Committees (03/103, 03/104, 18/EE/0032, 06/Q0108/84, 14/EE/0092, 20/N1/0109, 23/EM/0046) and conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent; children under the age of 16 provided written assent and their parents provided written

consent. Case 1's family were invited to participate in research studies at the Institute of Metabolic Science Translational Research Facility, Addenbrooke's Hospital, Cambridge, UK.

Genetic studies

DNA was extracted from whole blood of the proband (Case 1), both parents and the maternal uncle (Case 2). DNA was physically fragmented and prepared as libraries containing dual-indexed sequencing barcodes. The pre-capture libraries were enriched using SureSelect Human All Exon V6 capture baits (Agilent Technologies) for coding regions and splice junction sites of 20,000 human genes. The post-capture libraries were sequenced on a NovaSeq 6000 instrument (Illumina) with average read depth on target of 172x-184x and 97.3%-97.7% of target covered with at least 20x reads. The sequence data was analysed using a custom-developed bioinformatics pipeline (Novogene, UK), which aligned sequence data to the human genome (UCSC hg19), applied a GATK-based variant-calling pipeline (GATK v4.0.5.1) and annotated variants with respect to RefSeq transcripts using ANNOVAR. Variants were further annotated for Ensembl and RefSeq transcripts using Ensembl Variant Effect Predictor (VEP) v96/v109 (GRCh37/hg19).

Exome-wide variant prioritisation

Variant prioritisation was performed for possible autosomal recessive inheritance by searching for homozygous rare non-synonymous variants that were present in both Case 1 and Case 2 autosomal exomes (allele frequency < 2% in any of ExAC(7), gnomAD v2.1.1 genomes(8) and 1000Genomes v2015aug(9)). *GCGR* variants were subsequently confirmed in Case 1 and his parents by Sanger sequencing. Variant consequences for gene *GCGR* are reported with respect to transcript ENST00000400723.3.

Candidate gene panel for hepatic steatosis.

To define the candidate gene panel, we performed a PubMed™ search using the following terms: **1.** (“Non-alcoholic fatty liver disease” OR “Nonalcoholic fatty liver disease” OR “NAFLD” OR “nonalcoholic steatohepatitis” OR “NASH” OR “fatty liver” OR “hepatic steatosis”) AND (“gene” OR “genes” OR “genetic”) with the ‘Human’ filter on. **2.** (“steatosis”[Title/Abstract] OR “NAFLD [Title/Abstract])). ‘Other animals’ filter on. **3.** (“familial hypobetalipoproteinemia” AND “genes”) **4** (“familial hypobetalipoproteinemia” AND “SNP”). Abstracts were screened for relevance; relevant papers were screened for evidence, strength of evidence and relative references.

Clinical Studies

Anthropometry, Body composition, Respiratory Quotient. Weight and height were measured barefoot in light clothing. Dual X-ray absorptiometry (DEXA) (DPX software; Lunar Corp) was used to determine body composition. Respiratory quotient was determined by indirect calorimetry after an overnight fast using an open circuit, ventilated, canopy measurement system (Europa Gas Exchange Monitor; NutrEn Technology Ltd.).

Metabolic and Endocrine Tests. Liver function tests, lipid and amino acid profiles were measured in fasting samples. Plasma glucose, insulin, GLP-1 and FGF21 levels were measured in response to a 75 g oral glucose load over 120 minutes (insulin, GLP-1 and FGF21) or 240 minutes (glucose). Control samples were obtained from healthy individuals of normal BMI.

Abdominal imaging. MRI scans were carried out after a 4-hour fast on a 1.5-T whole-body system (GE Medical Systems) and included localizer images, T2-weighted fat-saturated

images, Proton Density Fat Fraction (PDFF), and Magnetic Resonance Elastography (MRE) as previously described(10).

Liver and pancreas biopsies. Liver and pancreas biopsies were obtained under clinical auspices using standard methods (Case 1 – percutaneous liver biopsy, Case 2 – percutaneous liver biopsy and liver explanted prior to orthotopic transplantation, Mother – endoscopic ultrasound guided biopsy of the pancreatic nodule and liver). Separate liver sections were stained with hematoxylin & eosin (H & E) and Picosirius red (PSR). Pancreas sections were stained separately with anti-glucagon antibody (Abcam, Cat No ab92517).

FGF21 Immunostaining Liver tissue for FGF21 immunostaining was collected from matched controls, who underwent a liver biopsy or liver transplant for clinical reasons and consented to the use of their biopsies for research and publication of results, with the approval of the Research Ethics Committee (20/NI/0109). Liver sections from cases and matched controls were stained with anti-FGF21 antibody (Abcam, Cat No ab66564) at a 1:150 dilution (11) and scanned using the Axio Scan.Z1 slide scanner (Zeiss) into whole digital images.

Molecular Studies

Cloning. The wild-type GCGR construct used in this study was made using human wild-type GCGR (NCBI NM_000160.5) ligated into pcDNA3.1(+)/N-(K)DYK vector (GenScript; Clone ID:OHu27939), or a C-terminal RlucII tag in pcDNA3.1(+). Single (R225H or V368M) and double (R225H; V368M) GCGR mutations were introduced by site-directed mutagenesis using QuikChange II XL kit (Agilent Technologies, 200516) according to the manufacturer's protocols and verified using Sanger sequencing.

Cell culture and transient transfection. HEK293 and HEK293SL cells(12) were cultured in Dulbecco Modified Eagle Medium (high glucose DMEM, GIBCO, 41965). Media were supplemented with 10% foetal bovine serum (GIBCO, 10270), 5% penicillin/streptomycin (Sigma-Aldrich, P0781) and 1% GlutaMAXTM (100x) (GIBCO, 35050) and maintained under 5% CO₂ atmosphere at 37°C in a humidified incubator. HEK293SL cells were transiently transfected with GCGR plasmid DNA using LipofectamineTM 2000 reagent (InvitrogenTM, 11668) in serum free Opti-MEM medium (GIBCO, 31985) as per the manufacturer's instructions.

Induced pluripotent stem cells (iPSCs). The KOLF2.1J iPSC line used in this study(13) was purchased from The Jackson Laboratory. Cells were recovered in mTeSRTM Plus medium (Stem Cell Technologies, Cat. No.100-0276) and maintained at 37°C and 5% CO₂ on 10 µg/ml Vitronectin XFTM (StemCell Technologies, 07180) coated six-well plates. Cells were passaged every 3-4 days using ReLeSRTM (StemCell Technologies, 100-0484).

Generation of GCGR^{R225H/V368M} iPSC line. iPSCs were electroporated using the Amaxa 4D electroporation system following the manufacturer's instructions. Briefly, 30.5 pmol guide RNA (gDNA; Synthego) was incubated with 30.5 pmol Cas9 nuclease (IDT, Acl-R S.p) to form ribonucleoprotein (RNP) complexes, followed by an equal amount of single stranded oligodeoxynucleotides (IDT). Separately, cells were harvested with TripLE Select (Gibco, Cat. No. 12563029), incubated with 5ml of mTeSRTM Plus medium containing CloneRTM2 supplement (Stem Cell Technologies, Cat. No.100-0691), centrifuged and resuspended in Nucleofector solution P3 containing Nucleofector Supplement (Lonza), then transferred to the Amaxa 4D-nucleofector for nucleofection. Cells were recovered at 32°C for 48h to promote homology directed repair (HDR). R225H gRNA: AGUCCAUCACCUGAGCGGAG; V368M

gRNA: UGGACUUCCUGUUUGAGAAG; R225H HDR Donor: ACACTGC ACC TGT ACC
AGG CGG TGG CTG GCT GCC ACG TGG CCG CGG TGT TCA TGC AAT ATG GCA TCG
TGG CCA; V368M HDR Donor: CTCTGCTGGGC GTC CAC GAA GTG GTC TTC GCC
TTC ATG ACG GAC GAG CAC GCC CAG GGC ACC CTG CGC TC.

Karyotype analysis.

Giemsa-band karyotyping was performed on > 20 iPSCs of each genotype by Cell Guidance Systems (cellgs.com).

Differentiation of iPSCs to iPSC-derived hepatocytes. iPSCs were differentiated to iPSC-derived hepatocytes as previously described(14) in Essential 6 Medium (ThermoFisher Scientific; days 1-2), RPMI-1640 Medium (Sigma-Aldrich; days 3-8) and HepatoZYME-SFM (Thermo Fisher Scientific, day 9 onward), supplemented with 3 μ M CHIR9901 (Day 1), 10 ng/ml BMP4 (Day 1-2), 10 μ M LY29004 (Day 1-2), 80 ng/ml FGF2 (Day 1-3), 100 ng/ml (day 1-3) and 50 ng/ml (Day 4-8) Activin A (Qkine, Cambridge), 10 ng/ml Oncostatin M (Day 9 onwards) and 50 ng/ml hepatocyte growth factor (HGF) (Day 9 -17). Day 21 iPSC-derived hepatocytes were dissociated using TrypLE Express Enzyme (ThermoScientific) and seeded on collagen-coated plates for downstream experiments.

Cell surface expression assay. Enhanced bystander bioluminescence resonance energy transfer (ebBRET) with rGFP-CAAX as the plasma membrane sensor, was used to test cell surface expression(15). HEK293SL cells were co-transfected with GCGR-RLucII and rGFP-CAAX plasmid DNA. The next day, culture medium was replaced by Tyrode's buffer. Total baseline fluorescence was measured, cells incubated for 1 hour at 37 °C to stabilise the signal, then incubated with 2.5 μ M luciferase substrate coelenterazine 400a – Deep Blue C (Nanolight

Technology) for 10 minutes. eBRET was measured on a TECAN M1000 Spark plate-reader equipped with acceptor filter (515 ± 30 nm) and donor filter (400 ± 70 nm). The ebBRET signal was determined as the ratio of the light emitted by rGFP-CAAX (energy acceptors) and the light emitted by RLucII-tagged receptors (energy donors). Values were expressed as percent of basal ebBRET obtained with wild-type GCGR. Data represent mean $\pm$ SEM from 5 independent experiments. Differences between WT and mutant receptors were compared using Welch's t-test.

Real-time cAMP production assay in HEK293 Cells. Real-time glucagon-induced cAMP production was measured in HEK293 cells transiently expressing WT or mutant GCGR using GloSensor™ cAMP biosensor (Promega) according to the manufacturer's instructions. Briefly, HEK293 cells were co-transfected with GCGR (WT or mutant, 20 ng/well) and pGloSensor™-20F cAMP reporter plasmid (Promega, E1171, 100 ng/well) using Lipofectamine 2000. After 24 hours, medium was replaced with 90 μ L of 2% v/v GloSensor cAMP reagent (Promega, E1290), and cells incubated for 2 hours at 37 °C. Basal luminescence was measured using a Spark M1000 plate-reader (Tecan), after which cells were treated with 10 μ L of 10x stock solution of increasing concentrations of human synthetic glucagon (Sigma-Aldrich, 05-23-2700). Real-time chemiluminescence was quantified for 30 minutes at 30 second intervals. In each experiment, a negative control using mock transfected cells (empty pcDNA3.1(+) plasmid) was assayed. The area under the curve (AUC) was calculated for each cAMP production curve considering total peak area above the baseline calculated as the average signal for mock pcDNA3.1(+)-transfected cells. For data normalization, the AUC from mock transfected cells was set as 0 and the AUC from WT GCGR was set as 100. Data are shown as sum curves from 4 independent experiments normalised to WT response $\pm$ SEM.

Establishing a response to Glucagon. iPSC-derived hepatocytes were seeded in collagen-coated 24-well plates at a density of 250,000 cells/well in HepatoZYME-SFM (ThermoFisher Scientific, 17705021) supplemented with non-essential amino acids (NEAA, Life Technologies, 11140-035), insulin-transferrin-selenium (Life Technologies, 41400045), Glutamax™ (Thermo Fisher Scientific, 35050061), chemically defined lipid concentrate (DLC, Thermo Fisher Scientific, 11905031) and penicillin/streptomycin. After 48 hours, cells were starved of glucagon in high-glucose Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, D5796) supplemented with NEAA, DLC, glutamate and oncostatin M, for 48 hours. They were then treated with 10^{-7} M glucagon for 30 mins or 24 hours and harvested for protein or RNA respectively.

cAMP production assay in iPSC-derived hepatocytes. iPSC-derived hepatocytes were seeded in black-walled, clear-bottom 96-well plates (Greiner, 655086). 24 hours before the assays, cells were changed to glucagon-free medium as described above and transduced with the Green Up cADDis cAMP sensor BacMam particles (Montana Molecular, U0250G). Prior to imaging, cells were washed once with PBS and incubated in KRBH buffer containing 6 mM glucose and 0.1% BSA and cells pre-incubated for 30 minutes at 37°C. Imaging was performed using an Nikon Ti2-E-based microscope (Cairn Research, UK) equipped with a LED source (CoolLED), 10x air objective, pco.edge 4.2 bi camera, ASI automated stage and heated enclosure (MicroscopeHeaters.com). Six epifluorescence fields-of-view per well acquired using a FITC filter set before and after addition of a range of concentrations of glucagon or vehicle. After 30 minutes, IBMX (3-isobutyl-1-methylxanthine, 500 μ M) plus forskolin (FSK, 50 μ M) were added to generate a maximal sensor response. To analyse changes in intracellular cAMP using ImageJ v1.54p (NIH), images were first subjected to flat field correction using BaSIC (16). A maximum intensity projection of each image time series was generated. Intensity

changes were measured and expressed relative to the IBMX/FSK-induced sensor maximum response after first subtracting baseline signal. Concentration responses from 5 independent experiments are expressed as area-under-curve, with 3-parameter logistic fitting performed using GraphPad Prism.

Western blotting. Cells were lysed in Pierce™ RIPA lysis and extraction buffer (Thermo Scientific, 89900) supplemented with protease and phosphatase inhibitors (Roche, 11836170001, 4906845001); samples were centrifuged and the supernatant used to quantify protein concentration using CD Protein Assay Kit (Bio-Rad, 5000112). 7.5ug of protein were used for SDS-PAGE. Proteins were transferred using the iBOLT-2 Invitrogen system to a PVDF membrane (IB24002X3), which was blocked with Protein-Free Blocking Buffer (Thermo, 3757), incubated with the primary antibody overnight at 4°C, and secondary antibody for one hour at room temperature, prior to developing using SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific, 34075) and imaged in Bio-Rad Chemidoc XRS. All antibodies were diluted in Protein-Free Blocking Buffer, anti-ERK (Total) (1:100, Cell Signal Technologies, Cat No. 4370L, Lot. 24), anti-pERK (1/2) (1:100, Cell Signal Technologies, Cat No. 4695S, Lot. 28), anti-GCGR (1:500, Abcam, Cat. No. Ab752400, Lot. GR3360811-12), anti-Vinculin (1:5000, Abcam, Cat. No. Ab129002, Lot. GR3270283-4), Goat anti-Rabbit IgG, HRP conjugated (1:2000, Thermo, G21234, Lot. 2321833). Band intensity was quantified using ImageJ.

Gene expression. Total RNA was isolated using a Total RNA Extraction Kit (NEB, T2010S) following the manufacturer's instructions. Reverse transcription and cDNA synthesis reactions were performed from 500 ng of total RNA using M-MLV Reverse transcriptase (Promega, M170A). cDNA amplification was performed using TaqMan probes and TaqMan Fast

Advanced Mix (Thermo, 444496)3 in a 10 μ L reaction volume according to the manufacturer's instructions. Samples were run in a duplex reaction. The gene of interest was detected using a FAM-labelled probe, while UBC or HMBS housekeeping genes were detected using a VIC-labelled probe. Results were analysed using the $\Delta\Delta$ CT method. Taqman probes (ThermoFisher): Albumin: Hs00609411_m1; HNF4A: Hs00230853_m1; SERPINA1: Hs00165475_m1; AFP: Hs01040598_m1; HMBS: Hs00609296_g1; UBC: Hs05002522_g1; GCGR: Hs01026191_g1; G6PC: Hs00609178_m1

Lipid accumulation assay. iPSC-derived hepatocytes were seeded in collagen-coated black/clear bottom 96-well plates (PhenoPlate™, PerkinElmer, 6055302), at a density of 50,000 cells/well, in HepatoZYME-SFM (ThermoFisher Scientific, 17705021) supplemented with non-essential amino acids (Life Technologies, 11140-035), insulin-transferrin-selenium (Life Technologies, 41400045), Glutamax™ (Thermo Fisher Scientific, 35050061) and penicillin/streptomycin. After 48 hours, cells were then treated with 0 – 480 μ M oleic acid (Sigma Aldrich, 01008-1G) conjugated with fatty acid-free bovine serum albumin (Sigma-Aldrich, 9048-46-8) in high glucose DMEM supplemented with non-essential amino acids and oncostatin M for 48 hours. Cells were then stimulated with -7 M glucagon in DMEM for 24 hours. Cells were then fixed with 4% formaldehyde and stained with 1:1000 BODIPY (Invitrogen D3922) and 1:5000 Hoechst 33342 (ThermoFisher Scientific). Each experiment was performed in triplicate. Wells were imaged using high content spinning confocal microscopy (Opera Phenix™, PerkinElmer) at 20 X magnification, 17 fields per well. Data were analysed using Harmony™ High-Content Imaging and Analysis Software (PerkinElmer). The lipid surface area was normalised to cell count. WT and mutant conditions were compared using a two-tailed student's t test. Data are shown as mean $\pm$ SEM from 7 independent experiments.

Statistical Analysis

Results were analysed using GraphPad Prism versions 9 and 10. For functional studies, the difference between mutant and wild-type was tested using a Student's t test. Welch's correction was adopted for groups with unequal variance, as indicated. All p values are from 2-sided statistical tests. $P < 0.05$ was considered statistically significant.

DATA AND RESOURCE AVAILABILITY

Requests for de-identified data relating to clinical studies may be addressed to the corresponding author (I.S.F.). Limitations on clinical data are designed to protect and respect patient and participant confidentiality. Resources are available upon request.

RESULTS

Exome sequencing

We performed whole exome sequencing of the proband (Case 1), his parents and the proband's uncle (Case 2). After filtering for rare variants (allele frequency $< 2\%$ in population databases), we examined all homozygous rare non-synonymous variants present in Case 1 and Case 2. We found that Case 1 and 2 were both homozygous for the same missense variants (R225H, V368M) in only one gene, *GCGR*, which encodes the Glucagon Receptor. The proband's mother was homozygous for both variants; the father was heterozygous for both variants (Supplementary Table S1). Each variant is very rare in publicly available exomes from people of European ancestry but more prevalent in people of South Asian origin: gnomAD v2.1(8) allele frequency for R225H: 0.0017% in Europeans, 0.18% in South Asians; for V368M 0.0069% in Europeans, 0.027% in South Asians. Here the two variants occurred on the same allele, representing a haplotype that is more prevalent in people of South Asian ancestry

(gnomAD v2.1 (8): double variant was heterozygous in 4, and homozygous in 1, out of 11,363 exomes from people of South Asian ancestry; absent in people from all other ancestries). In addition to exome-wide variant prioritisation, we assessed all variants in a panel of 93 existing candidate genes for hepatic steatosis. We found common and rare variants in other steatotic liver disease-associated genes; however, none co-segregated with hepatic steatosis in this family (Supplementary Table S1).

Plasma glucagon concentrations were 60-100 times above the upper limit of normal in homozygous variant carriers (Table 1), in keeping with a diagnosis of Glucagon receptor deficiency. Fasting glucagon levels fell markedly after transplantation in Case 2 (pre-transplant 1213 pmol/L; post-transplant 75 pmol/L; normal range 0–50 pmol/L).

Metabolic studies and abdominal imaging

We performed metabolic studies to characterize the features of GCGR deficiency. The mother of Case 1 was normal weight (BMI 21 kg/m²) with increased liver fat measured by MRI proton density fat fraction (PDFF) and confirmed on liver biopsy. MRI revealed pancreatomegaly, with a smooth pancreatic contour, normal parenchyma, no hyper- or hypo-enhancing rim and no vascular involvement as seen previously (4, 5). In addition, there was a 7 mm nodule in the tail of the pancreas (Figure 2A); fine needle aspiration revealed pancreatic islets staining heavily for glucagon indicative of alpha cell hyperplasia, without neoplastic transformation (Figure 2B). There was no radiological or histological evidence of porto-sinusoidal vascular disease (PSVD) or portal hypertension.

Serum concentrations of total Glucagon-like peptide 1 (GLP-1) were markedly elevated in cases compared to controls (Figure 2C). Further characterisation by LC-MS showed that excess

GLP-1 consisted predominantly of the inactive pancreatic derived peptides, GLP-1 1-37 and 1-36.

Phenotypic studies of Case 1 and his mother, using dual X ray absorptiometry (DEXA) and abdominal MR imaging, showed that both had increased fat mass compared to control individuals matched for age, sex and BMI, which was predominantly in the subcutaneous (rather than visceral) compartment (Figure 2D). While fasting plasma glucose and insulin were within the normal range (Table 1), insulin secretion was markedly increased in response to a 75g oral glucose load (Figure 2E-F). Serum triglycerides were relatively low, compared to healthy controls and individuals with Metabolic dysfunction-associated steatotic liver disease (MASLD, Table 1). In Case 1, respiratory quotient measured by indirect calorimetry was reduced compared to 44 control individuals matched for age, sex and BMI ($0.7 \text{ v } 0.84 \pm 0.04$, mean $\pm$ SD; $p < 0.05$) suggesting impaired fat oxidation.

Circulating levels of the liver derived hormone Fibroblast Growth Factor 21 (FGF21) are 25-fold higher in *Gcgr* knockout mice than in wild-type mice(17). We found that both GCGR deficient individuals had markedly elevated fasting FGF21 levels which remained elevated during a 2-hour oral glucose tolerance test compared to 8 healthy individuals matched for BMI (mean BMI = 22.1 kg/m²) (Figure 2G). As both mouse and human liver single cell RNA sequencing datasets, indicate that FGF21 expression is restricted to hepatocytes (<https://www.livercellatlas.org/umap-humanAll.php>), increased circulating FGF21 levels may be a sign of hepatocyte stress and injury(18). Immunohistochemistry on liver samples showed that hepatic FGF21 expression was increased in homozygous GCGR variant carriers compared to individuals with a normal liver, and comparable to individuals with MASLD (Supplementary Figure S2 and Supplementary Table S2).

Functional characterization of *GCGR* mutations

We investigated the functional consequences of the double mutation in *GCGR*, which is a membrane-expressed $G_{\alpha s}$ -coupled receptor whose activation results in increased intracellular cyclic AMP (adenosine monophosphate) levels. Using a bioluminescence resonance energy transfer (BRET) assay, we found that whilst V368M *GCGR* did not alter cell membrane expression, R225H *GCGR* and the double mutant (*GCGR*^{R225H/V368M}) significantly reduced cell membrane expression compared to wild type (WT) *GCGR* (Figure 3A). Using a cAMP biosensor (GloSensor™), the double mutant abolished glucagon-dependent cAMP production (Figure 3B) compared to WT, consistent with a complete loss of function. In a previous study, these two mutant forms of *GCGR* were shown to impair ligand binding in cells(19).

To test whether these loss-of-function *GCGR* mutations can directly cause lipid accumulation, we used human induced pluripotent stem cell-derived hepatocytes (iHEPs) which have been used to model several inherited metabolic disorders of the liver (20). We used the previously validated human induced pluripotent stem cell line (iPSC, KOLF2.1J) (13) to generate a *GCGR* R225H/V368M knockin line (*GCGR*^{R225H/V368M}) using CRISPR-Cas9 technology (Figure 4, Supplementary Figure S3). WT and mutant iPSCs were differentiated to hepatocytes and validated using established protocols (14) (Figure 5). We confirmed that *GCGR* was expressed in differentiated iHEPs using Western blot and qPCR (Supplementary Figure S4A-B), showed WT iHEPs respond to glucagon and that this signaling response is impaired in *GCGR*^{R225H/V368M} mutant cells (Supplementary Figure S5). Similarly, we showed that WT iHEPs generate cAMP in response to glucagon and that this response was abolished in *GCGR*^{R225H/V368M} cells (Figure 3C). We found that stimulation of WT iHEPs with oleic acid leads to a dose-dependent increase in intrahepatocellular lipid accumulation, which is increased 2-fold in *GCGR*^{R225H/V368M} mutant

cells compared to WT, most prominent in the absence of supplementary oleic acid (Figure 6A-C). Collectively, these experiments show that a severe loss of function double mutation in GCGR causes increased lipid accumulation in a cellular model of human steatosis.

DISCUSSION

In summary, we extend the clinical phenotype of GCGR deficiency (also referred to as Mahvash Disease) to include hepatic steatosis and increased adiposity despite normal body weight, alongside known features which include, elevated glucagon levels, alpha cell hyperplasia (and/or pancreatic nodules) and elevated levels of glucogenic amino acids, likely due to their reduced hepatic uptake and catabolism(21). We recommend that measuring glucagon levels and genetic analysis is considered in people who have early-onset hepatic steatosis, especially those with pancreatomegaly, in the absence of obesity and those of South Asian ancestry in whom there is a high prevalence of monogenic diseases (22).

Here, by characterising a human severe loss-of-function *GCGR* mutation, we demonstrate that impaired *GCGR* signaling causes hepatic steatosis. Our findings align with experiments in *Gcgr* knockout mice, rats and zebrafish, which exhibit steatosis, hepatocyte injury and raised FGF21. This phenotype has been observed under standard diet conditions, but is exacerbated with a high fat diet (HFD) or the Gubra-Amylin NASH (GAN) diet (23-25). Detailed transcriptomic and metabolomic profiling in mice and zebrafish (fed a standard diet) showed reduction in fatty acid oxidation and upregulation of fatty acid and cholesterol synthetic pathway (26; 27).

Drucker and colleagues proposed that GCGR signaling above a certain threshold is required for hepatocyte survival(28). It is likely that the severe functional impairment caused by the

double GCGR mutation studied here falls below such a threshold, leading to steatosis, hepatic injury (characterised by raised FGF21 levels) and potentially cirrhosis, as suggested by Case 2. This threshold effect may explain why hepatic steatosis has not to date been reported in other GCGR mutation carriers, nor in association studies of common *GCGR* variants in the population.

This is the second reported case of liver transplantation in GCGR deficient individuals. As in the previous report, we found that restoring GCGR activity with transplantation results in reduced size of the pancreatic lesions and a reduction in plasma glucagon concentrations (29). Robbins and colleagues report the presence of portal hypertension in the absence of cirrhosis, with features of sinusoidal dilatation on liver biopsy. The latter was not a feature in any of our three cases; the reasons behind this variation are unclear.

Glucagon receptor antagonists have been trialled in patients with type 2 diabetes in whom elevated glucagon levels contribute to hyperglycemia (30; 31). However, dose-dependent increases in alanine transaminase and hepatic fat content in people treated with a GCGR antagonist, suggest that glucagon may have clinically significant effects on human lipid metabolism. While development of some GCGR antagonists has been discontinued, increased vigilance and surveillance of participants is recommended when trialling therapies which impair GCGR signaling. In contrast, GCGR agonists are likely to reduce hepatic steatosis as predicted by genetic disruption in mice and now by our studies in humans. Indeed, trials of GCGR agonists in combination with GLP-1R and/or GIP receptor agonists have reported improvements in steatosis after 48 weeks in people with obesity and MASH(32). Collectively, these results indicate that the relative potency of agonists at the receptors for Glucagon and GLP-1 may explain the degree to which they impact body composition and hepatic steatosis.

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Author Contributions

T.M.C., E.H., J.M.K., R.B., M.S., D.H., L.W., M.H., A.B. and I.S.F. were involved in recruiting cases and controls, clinical studies and analysing clinical data. K.L. performed genetic analysis. T.M.C., K.M.M.A., E.M.O., A.S., D.A., B.J., T.R, performed the functional characterisation of human mutations in cells and stem cell-derived hepatocytes. T.M.C. and A.D. performed histological analysis of human samples. T.M.C. and E.M.G. analysed MRI data. I.S.F. is the guarantor of this work, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final manuscript.

Duality of interest

I.S.F. has consulted for a number of companies developing weight loss drugs (including Eli Lilly, Novo Nordisk, and Rhythm Pharmaceuticals) and investors (Goldman Sachs, SV Health). B.J. has received funding from Eli Lilly, Metsera Inc and Sun Pharmaceutical Industries, and acts as a consultant for Metsera Inc. None of the authors have any conflicts to declare.

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Table 1: Clinical and Biochemical measurements in Glucagon Receptor deficient family members

<i>GCGR</i> mutations	Case 1	Mother	Father	Controls
R225H; V368M	Homozygous	Homozygous	Heterozygous	
Age (years)	15.5	39.1	49.4	
Sex	M	F	M	

BMI (kg/m ²) [sds]	21.1 [0.6]	22.1	27	
DEXA Fat Mass (%)	37.7	42.8	34.4	
MRI Proton Density Fat Fraction (<6.5 %)	18.4	23	13	
MR Elastography (<3.3 kPa)	2.2	1.59	2.54	
Bilirubin (0-20 µmol/L)	17	9	17	
Alanine Aminotransferase (10 – 49 U/L)	48	88	35	
Aspartate Transaminase (≤ 34 U/L)	27	52	17	
Alkaline Phosphatase (60 – 425 U/L)	221	67	74	
LDL-Cholesterol (≤3 mmol/L)	1.52	2.11	2.75	
Triglycerides (0.3 – 1.8 mmol/L)	0.44	0.43	0.97	
Apolipoprotein B (0.6 – 1.3 g/L)	0.5	0.6	0.9	
Glycated hemoglobin (< 48 mmol/mol or 6.5%)	36 (5.4%)	39 (5.7%)	98 (11.1%)	
Glucose (3.5 – 6.0 mmol/L)	4.2	4.7	11.8	
Insulin (0 – 60 pmol/L)	39	39	47	
Glucagon (0 – 50 pmol/L)	2919	4596	9	
FGF21 (pg/mL)	625	1507	428	170
Plasma amino acids				
Alanine (155 – 537 µmol/L)	664	822	660	
Arginine (49 – 129 µmol/L)	249	204	46	
Aspartate (0-14 µmol/L)	9	6	6	
Citrulline (8-47 µmol/L)	57	58	15	
Glutamate (8-64 µmol/L)	88	79	87	
Glutamine (464-728 µmol/L)	1460	1051	492	
Glycine (160-304 µmol/L)	343	292	172	
Histidine (65-105 µmol/L)	105	98	85	

Isoleucine (41 – 93 µmol/L)	94	58	88	
Leucine (85-169 µmol/L)	169	117	162	
Lysine (142 – 198 µmol/L)	322	243	262	
Methionine (17 – 37 µmol/L)	34	30	27	
Ornithine (21 – 77 µmol/L)	148	131	34	
Phenylalanine (42 – 74 µmol/L)	55	48	62	
Proline (70 – 300 µmol/L)	482	305	281	
Serine (70 – 178 µmol/L)	250	178	104	
Taurine (0 – 232 µmol/L)	69	57	66	
Threonine (75 – 203 µmol/L)	452	295	177	
Tyrosine (40 – 94 µmol/L)	98	105	67	
Valine (161 – 285 µmol/L)	289	207	306	

Footnotes: For measurements where normative data is not available, we recruited age, sex and BMI-matched controls from the local population (FGF-21 n=8). Abbreviations: BMI – body mass index, sds – standard deviation score, DEXA – dual energy X-ray absorptiometry, MRI – magnetic resonance imaging, LDL – low density lipoprotein. Results shown in bold are outside the reference range.

Table 2. Biochemical investigations performed in Case 1.

	Parameter (Reference Range)	Case 1
Haematology	White Cell count (4.2 – 10.8 x10 ⁹ /L)	5.9
	Haemoglobin (130 – 166 g/L)	131
	Mean Cell Volume (79.0 – 96.0 fL)	80.8
	Mean Cell Haemoglobin (26.5 – 33.0 pg)	27.1
	Platelet Count (160 – 385 x10 ⁹ /L)	323
Coagulation	Prothrombin Time (10.3 – 12.9 seconds)	13.3
	Activated Partial Thromboplastin Clotting Time (26.7 – 35.7 seconds)	32.6

Biochemistry	Sodium (133 – 146 mmol/L)	139
	Potassium (3.5 – 5 mmol/L)	4.2
	Urea (2.5 – 6.5 mmol/L)	4.5
	Creatinine (34 – 71 $\mu\text{mol/L}$)	37
	Albumin (30 – 50 g/L)	40
	Adjusted Calcium (2.2 – 2.7 mmol/L)	2.51
	Phosphate (0.9 – 1.8 mmol/L)	1.67
	Amylase (12 – 118 U/L)	37
	Lipase (6 – 51 U/L)	36
	Creatine Kinase (43 – 178 U/L)	53
	Tissue transglutaminase Ig A antibody (0.0 – 6.9 μmol)	0.4
	Folate ($\geq 5.38 \mu\text{g/L}$)	4.46
	Serum osmolality (275 – 295 mOsm/kg)	284
	Alpha-Feto Protein (<4 kIU/L)	<2 kIU/L
Virology	Hepatitis B Surface Antigen	Not detected
	Hepatitis C antibody	Not detected
Plasma bile acids	Glycodihydroxycholanoate (0.0 – 5.98 $\mu\text{mol/L}$)	1
	Glycotrihydroxycholanoate (0.0 – 1.9 $\mu\text{mol/L}$)	<1
	Taurodihydroxycholanoate (0.0 – 1.9 $\mu\text{mol/L}$)	<1
	Taurotrihydroxycholanoate (0.0 – 1.9 $\mu\text{mol/L}$)	<1
Endocrine	Follicular stimulating hormone (1.0 – 10.1 U/L)	3.0
	Luteinising hormone (1.5 – 6.3 U/L)	3.9
	Thyroid stimulating hormone (0.35 – 5.5 mU/L)	1.33
	Free Thyroxine (10.5 – 21.0 pmol/L)	20.4
	Free Triiodothyronine (4.2 – 7.5 pmol/L)	6.7
	Sex hormone binding globulin (10 – 57 nmol/L)	24
	Insulin-like growth factor 1 (23.0 – 65.9 mmol/L)	44.7
	Insulin-like growth factor binding protein 3 (2.6 – 6.3 mg/L)	4.3
	Testosterone (10 – 35 nmol/L)	16.8
Metabolic	Ammonia (<50 $\mu\text{mol/L}$)	44
	Lactate (<1 mmol/L)	0.9
	Caeruloplasmin (0.20 – 0.50 g/L)	0.27
	Urine copper (24 hours post penicillamine) (0.0 – 12.0 $\mu\text{mol/24 h}$)	10.56
	Alpha-1 antitrypsin	Normal
	Free carnitine (10 – 45 $\mu\text{mol/L}$)	32
	Urine organic acids	Normal
	Porphobilinogen creatinine ratio (0.0 – 1.5 $\mu\text{mol/mmol Creat}$)	0.5
	Immunoglobulin M (IgM) (0.5 – 1.9 g/L)	1.25
	Immunoglobulin G (IgG) (6.0 – 16 g/L)	7.32

Immunology	Immunoglobulin A (IgA) (0.8 – 2.9 g/L)	1.21
	Mitochondrial antibody	negative
	Liver kidney muscle antibody	negative
	Anti-smooth muscle antibody	negative
Very long chain fatty acids	C22 (31 – 98 µmol/L)	63.75
	C24 (24 – 66 µmol/L)	52.99
	C26 (0.15 – 0.91 µmol/L)	0.44
	C24/C22 (0.00 – 0.96)	0.83
	C26/C22 (0.000 – 0.022)	0.007
	Phytanate (0.00 – 15 µmol/L)	2.07
	Pristanate (0.00 – 2.00 µmol/L)	< 0.33

Footnotes: International system of Units (SI) are used and reference ranges given.

FIGURE LEGENDS

Figure 1. A homozygous double mutation in the gene encoding the Glucagon receptor identified in a consanguineous family with early-onset hepatic steatosis.

A. Pedigree of the family with *GCGR* deficiency: proband (Case 1) indicated by an arrow, black filled symbols indicate homozygous variant carriers, heterozygotes shown in grey, empty symbols denote unknown genotypes, squares denote males, circles denote females, double lines indicate consanguineous marriages. **B.** Percutaneous liver biopsy from a 27-year-old male homozygous for *GCGR*^{R225H/V368M} stained using haematoxylin & eosin; black arrow – macrovesicular steatosis; red arrow – ballooned hepatocytes; green arrow – chronic inflammatory infiltrate; magnification 10X. **C.** 15 mm nodule in the head of the pancreas (white arrow) in *GCGR*^{R225H/V368M} mutation carrier (Case 2). **D.** Explanted liver of *GCGR*^{R225H/V368M} mutation carrier aged 50 (Case 2) demonstrating macronodular cirrhosis; stain Picosirius red, magnification: 5x.

Figure 2. Metabolic phenotype of *GCGR* variant carriers.

A. Magnetic Resonance Imaging of the proband's mother depicting a 7 mm nodule in the tail of the pancreas (arrow). **B.** Glucagon immunostaining of the pancreas obtained from a *GCGR* mutation carrier (left) and healthy control (right). Brown = Glucagon, Blue = Nuclei, Scale bar = 100 μ m. **C.** Plasma GLP-1 concentrations measured before and during an oral 75 g glucose load in Case 1 (black squares), his mother (black circles) and matched controls (open circles). n = 6; mean $\pm$ SD shown. **D.** MRI measurement of abdominal fat distribution at the level of the L3/L4 intervertebral disc analysed using Osirix™ to differentiate subcutaneous (green) and visceral (red) fat, in Case 1 (left) and his mother (right). **E-G.** Plasma insulin (E), glucose (F) and FGF21 (G) concentrations were measured after the oral administration of 75 g glucose

(Time 0) in Case 1 (black, closed squares), Mother (black, closed circles) and 8 controls matched for age, sex and BMI (grey, open circles).

Figure 3. Double mutation in the Glucagon Receptor causes a loss of function in cells.

Functional studies of wild-type (WT) and mutant forms of GCGR. **A.** Cell surface expression in the enhanced bystander bioluminescence resonance energy transfer (BRET) assay expressed as a percentage of WT in each experiment; n=5, mean $\pm$ SEM shown. **B.** GCGR-mediated cAMP production in response to glucagon stimulation in HEK293 cells, expressed as % WT; n=4, mean $\pm$ SEM. **C.** GCGR-mediated cAMP production in iPSC-derived hepatocytes, expressed as area under the curve (AUC); n=5, mean $\pm$ SEM.

Figure 4. Generation of homozygous knockin human induced pluripotent stem cell line

***GCGR*^{R225H/V368M} using CRISPR/Cas9**

Generation of *GCGR*^{R225H/V368M} hiPSC line. **A.** CRISPR/Cas9 strategy targeting exons 8 and 12 to generate the mutant model. White boxes = non-coding exons; Blue boxes = coding exons; Purple residues = Protospacer adjacent motif (PAM) sequence; Blue residues = Target sequence; Green = Single-stranded oligodeoxynucleotides (ssODN); Red residues = Mutated residues. **B.** Chromatograms showing sequencing analysis to confirm the presence of the two targeted mutations. Figure 4B was prepared using Geneious Prime. gRNA = guide RNA; HDR = homology directed repair; RNP = ribonucleoprotein; ssODN = single stranded oligodeoxynucleotides.

Figure 5. iPSC differentiation to iPSC-derived hepatocytes.

A. Summary of four-stage differentiation protocol from human iPSCs to definitive endoderm, hepatic progenitor and hepatocytes over a 21-day period, using a combination of growth factors

and small molecules indicated in blue. **B.** Brightfield microscopy using EVOS cell imaging system, depicting typical hepatocyte morphology with characteristic polyhedral appearance; (Left) GCGR Wild type, (Right) GCGR^{R225H/V368M}; scale bar = 100 μ m. **C.** Expression of canonical hepatocyte markers (Albumin, HNF4A1, SERPINA1) in GCGR WT and R225H/V368M hepatocytes relative to the housekeeping gene HMBS. N = 6-7 experiments. Mean $\pm$ SEM.

Figure 6. Increased lipid accumulation in GCGR R225H/V368M human induced pluripotent stem-cell derived hepatocytes.

A. Lipid accumulation in GCGR^{R225H/V368M} human induced pluripotent stem-cell (iPSC)-derived hepatocytes (bottom row) compared to WT GCGR (top row) using confocal microscopy; BODIPY staining of neutral lipid (green), Hoechst 33342 staining of nuclei (blue), scale bar, 50 μ m. **B.** Lipid accumulation measured as lipid surface area divided by number of nuclei in WT GCGR (white) and GCGR^{R225H/V368M} (grey) iPSC-derived hepatocytes treated with increasing concentrations of oleic acid; data acquired using confocal microscopy and quantified using Harmony High-Content Imaging and Analysis Software; n=7, mean $\pm$ SEM, ns-not significant; 2-tailed student's t test.

Figure 1

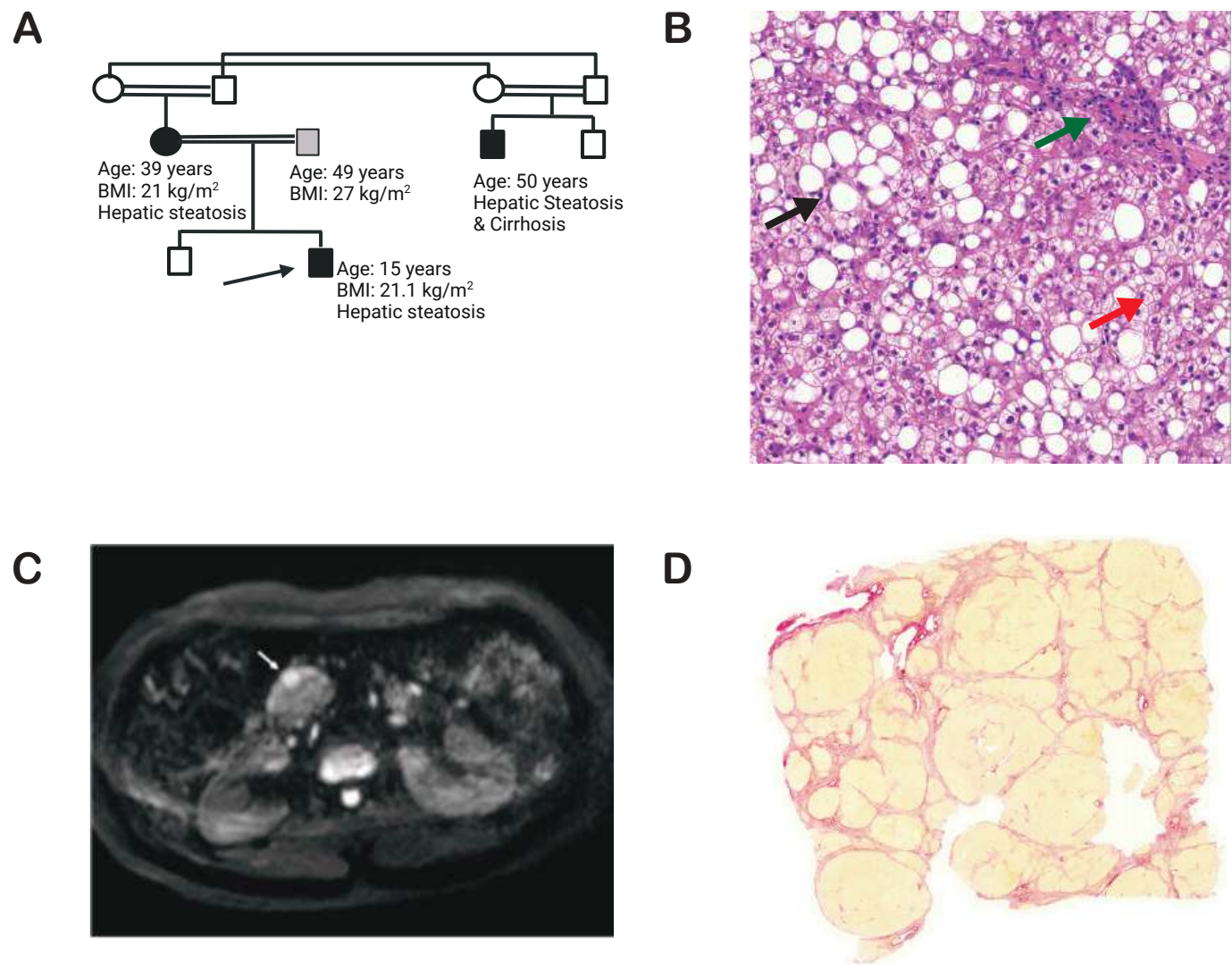


Figure 2

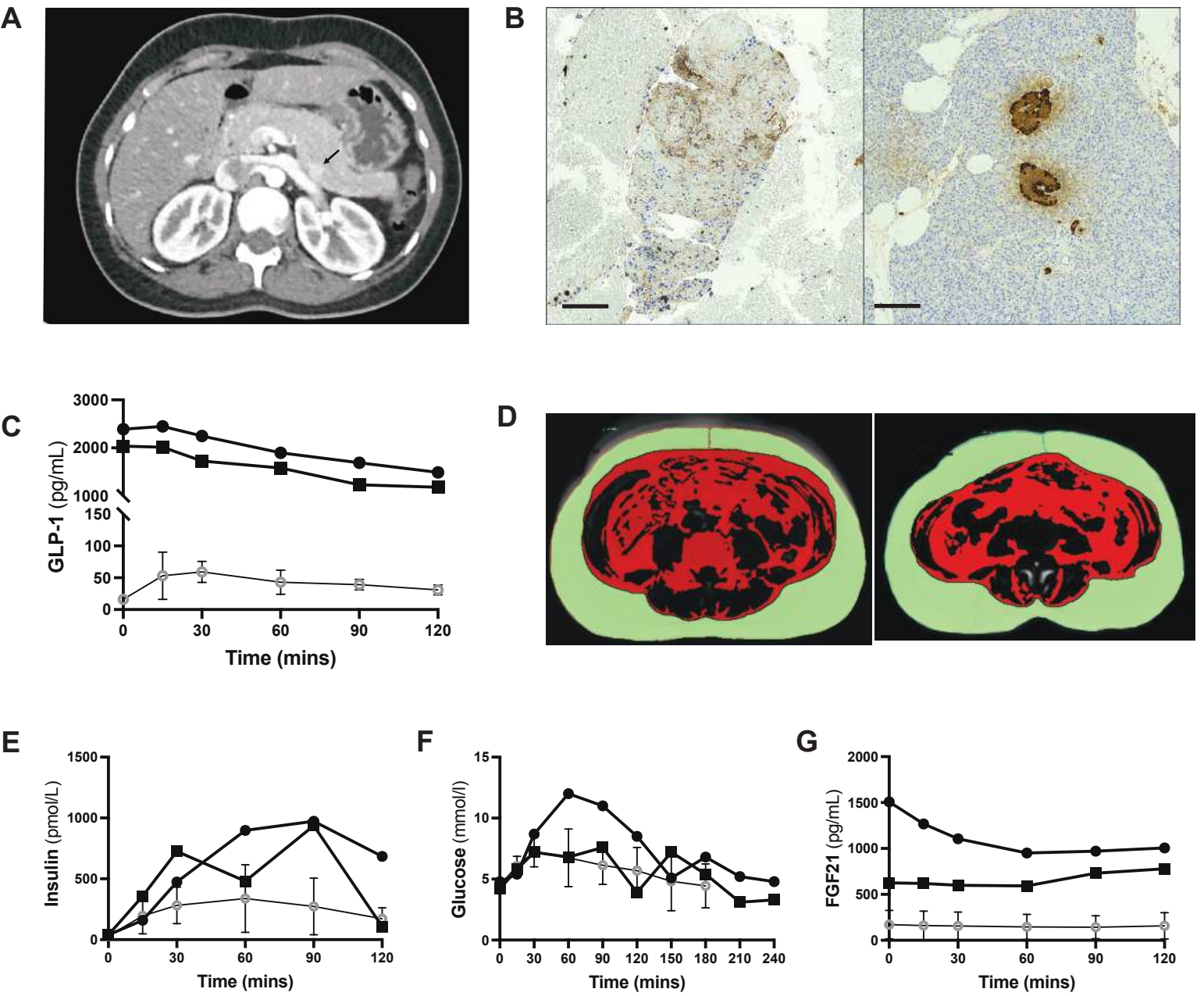


Figure 3

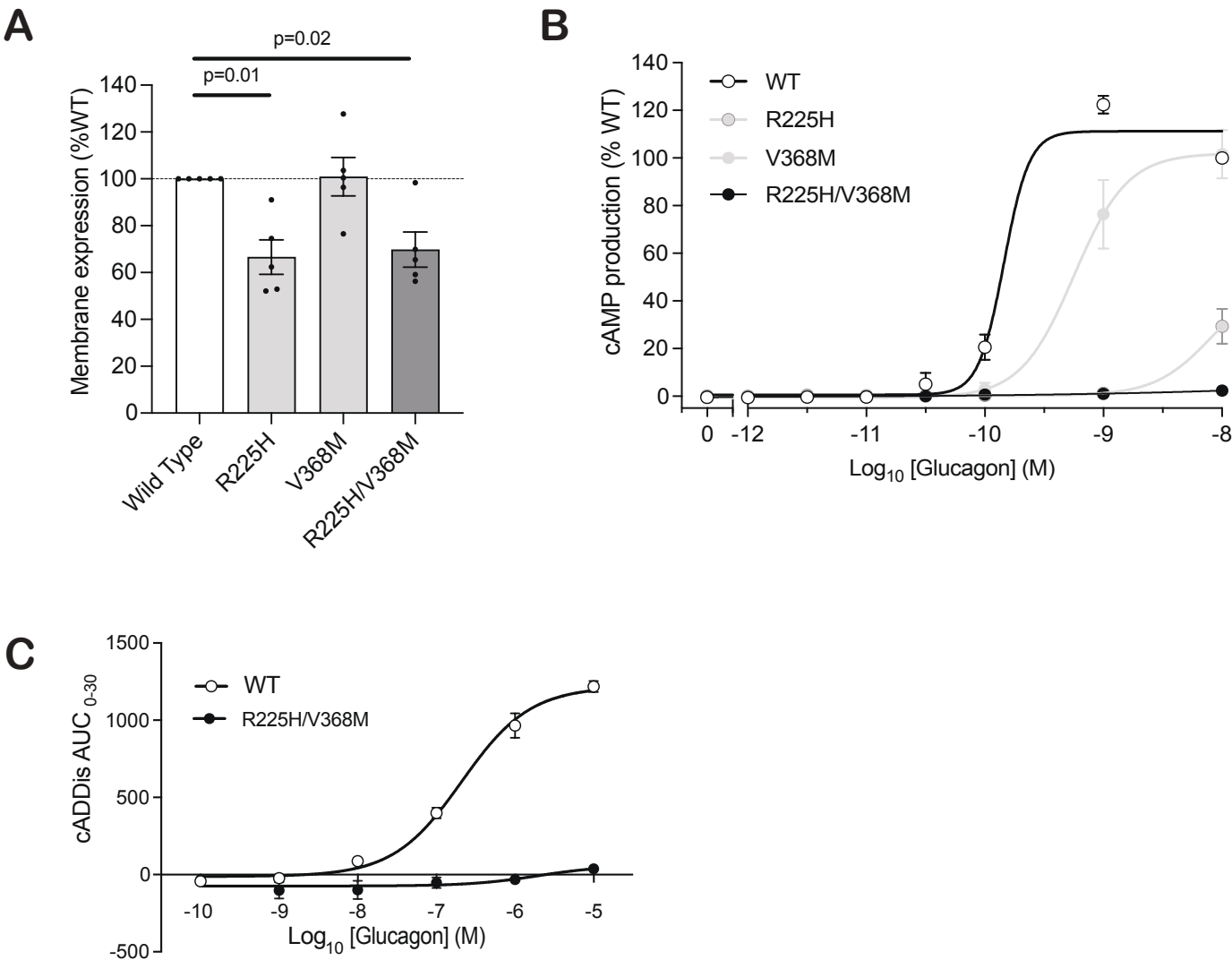


Figure 4

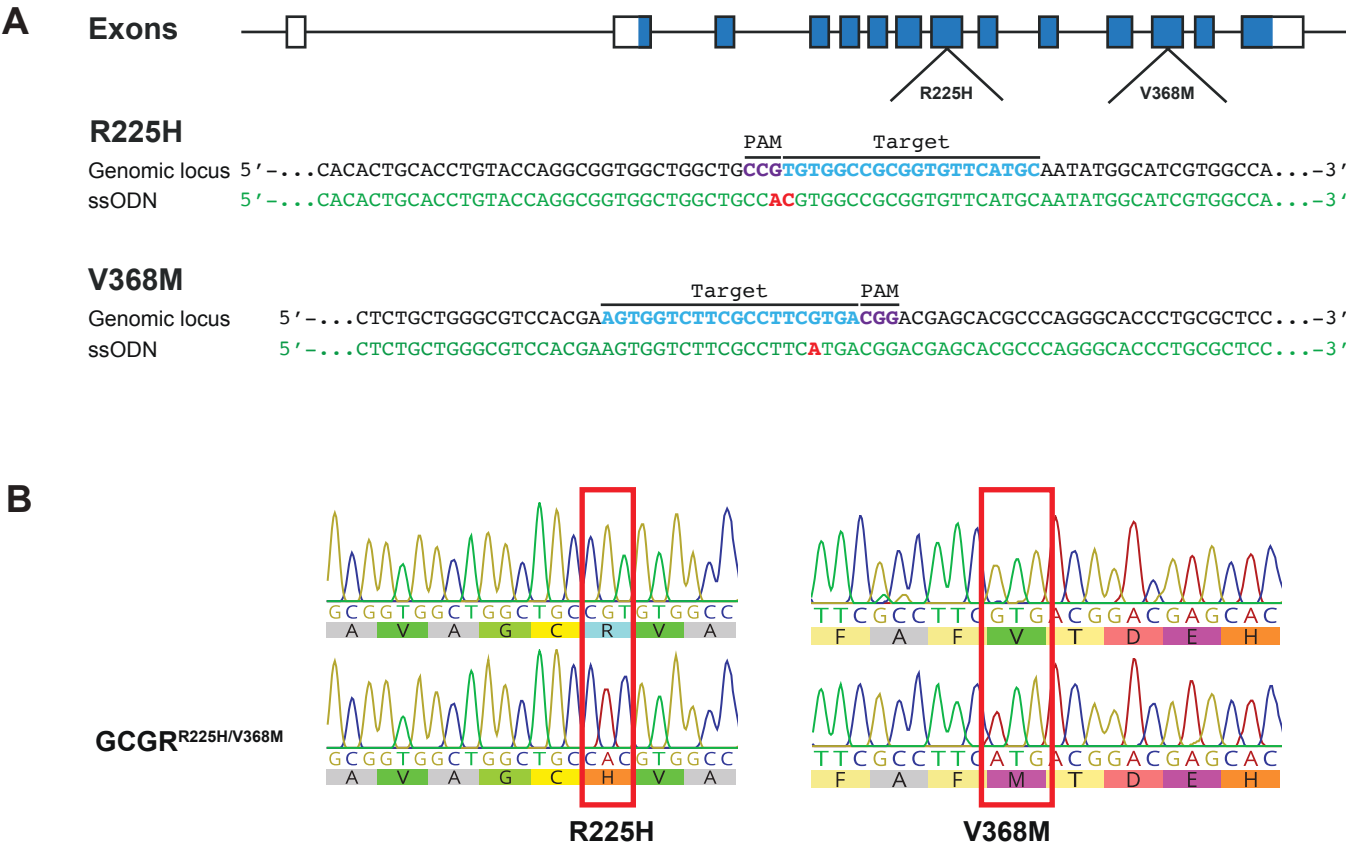


Figure 5

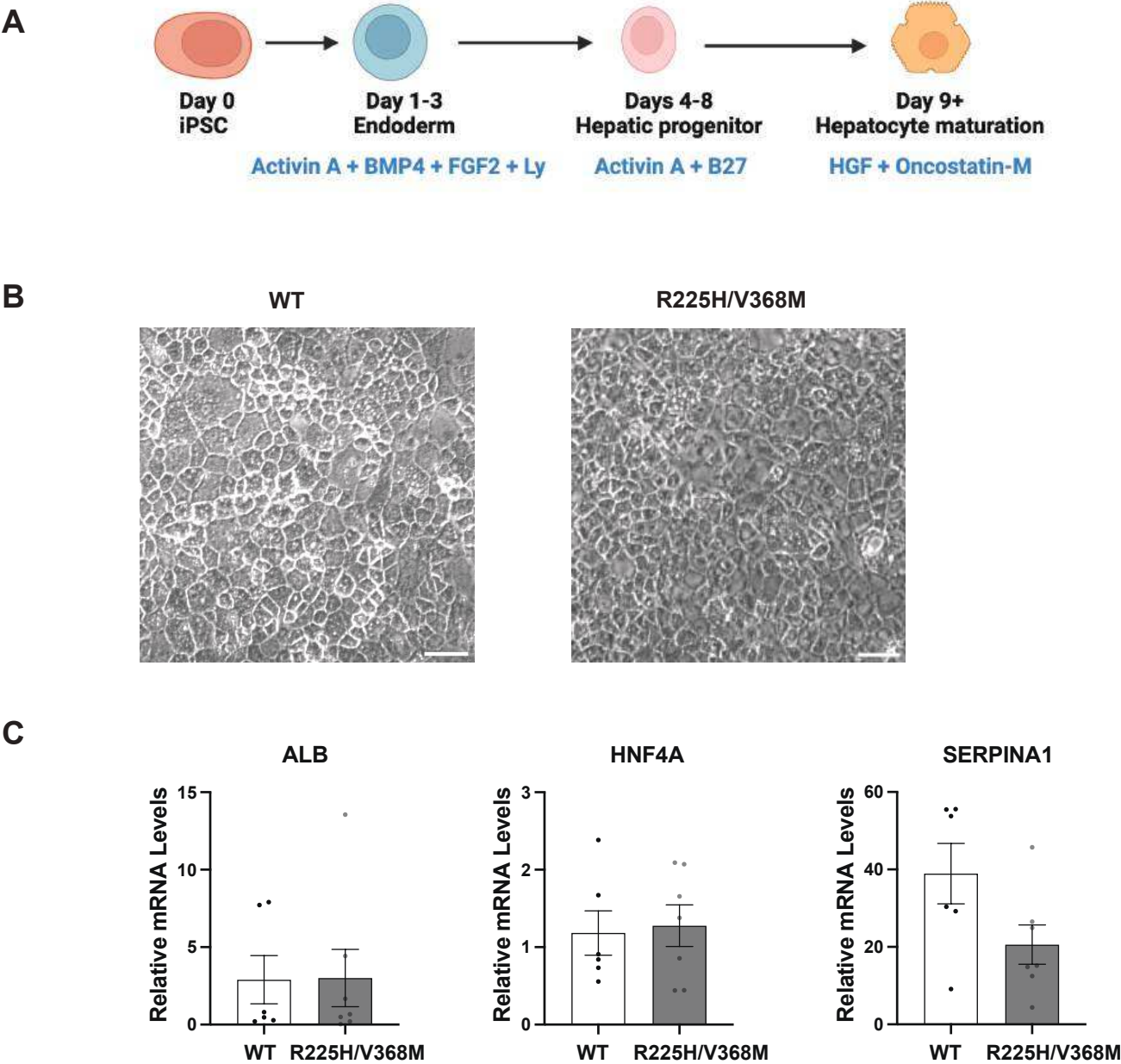
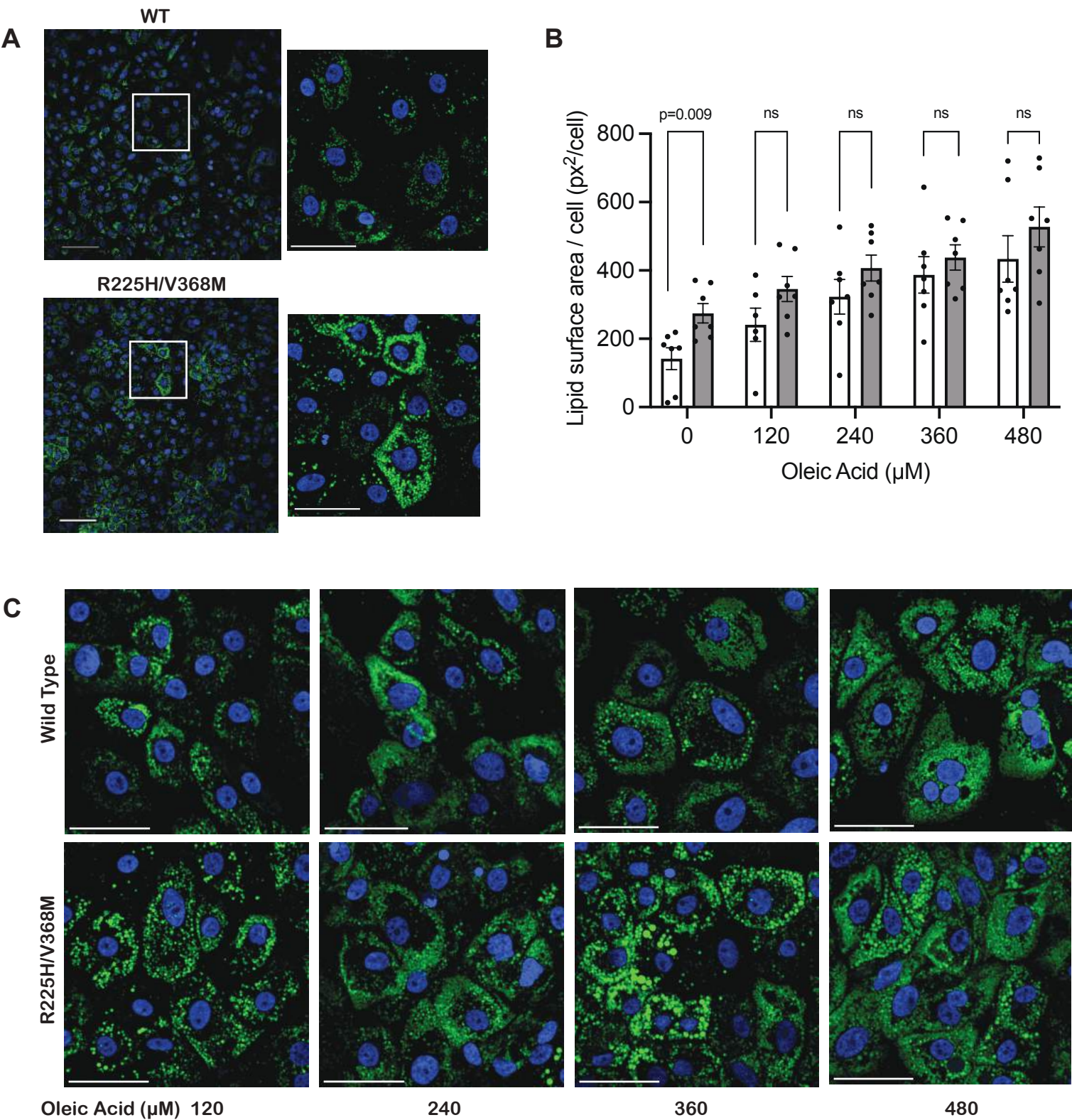
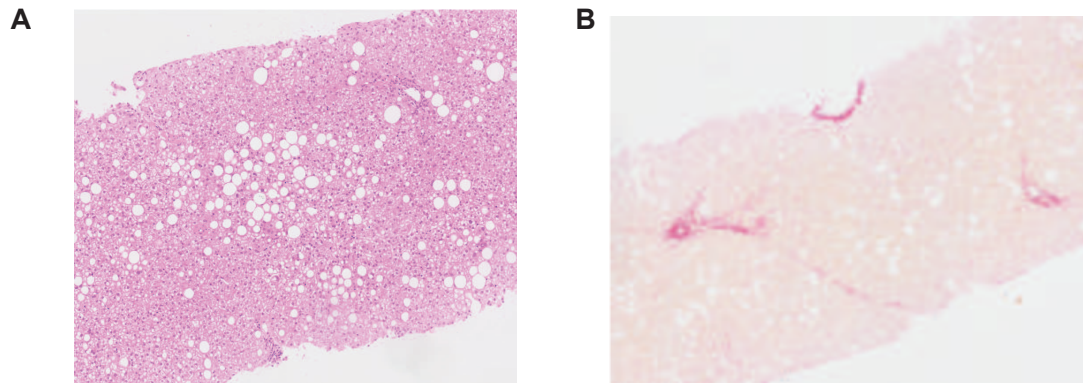


Figure 6



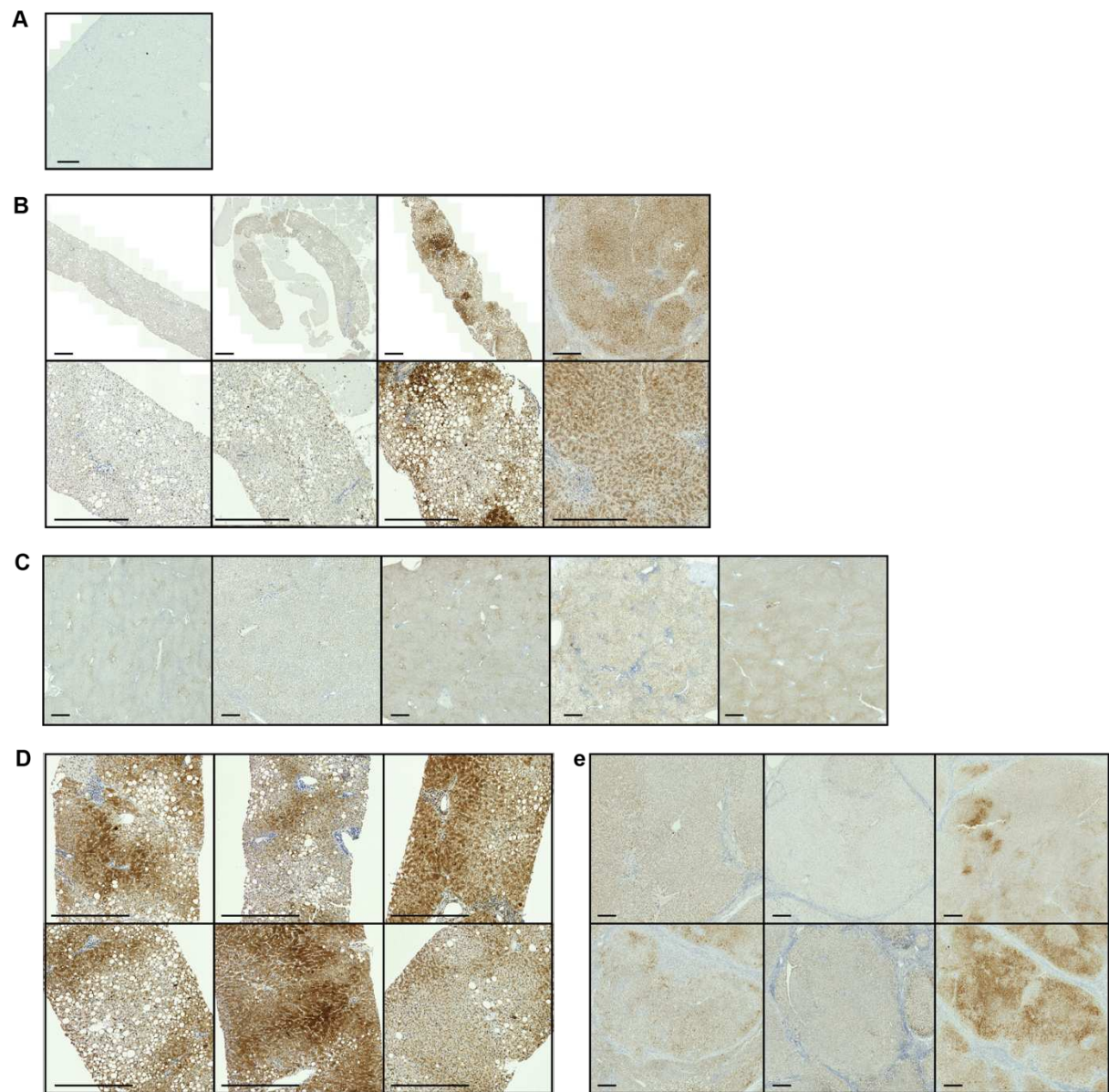
Supplemental material: **Glucagon receptor deficiency causes early-onset hepatic steatosis**

Supplementary Figure S1. Liver biopsies from *GCGR* mutation carriers (Case 1)



Percutaneous liver biopsies obtained from Case 1 at the age of 8 years. Panel A: H&E staining, 10X magnification, Panel B: Picosirius red staining for fibrosis, 5X magnification.

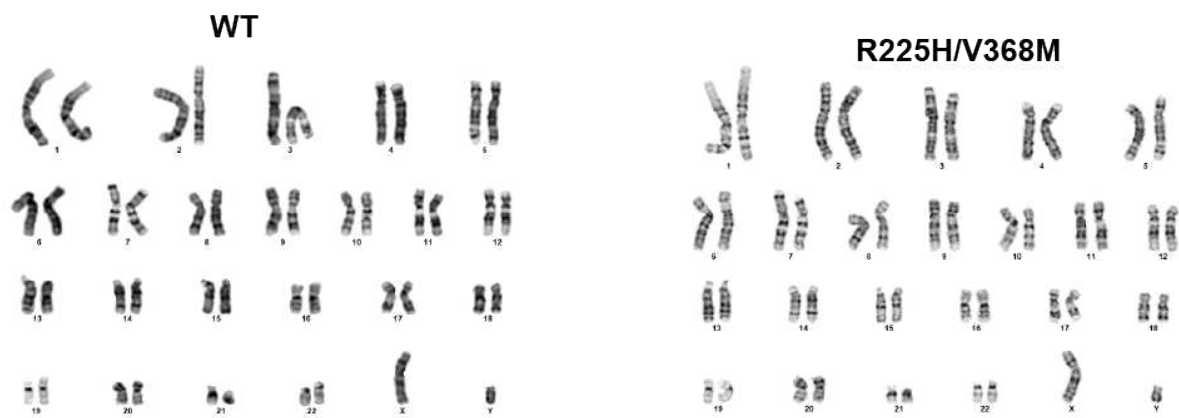
Supplementary Figure S2. FGF21 immunostaining of liver tissue in *GCGR* mutation carriers and controls.



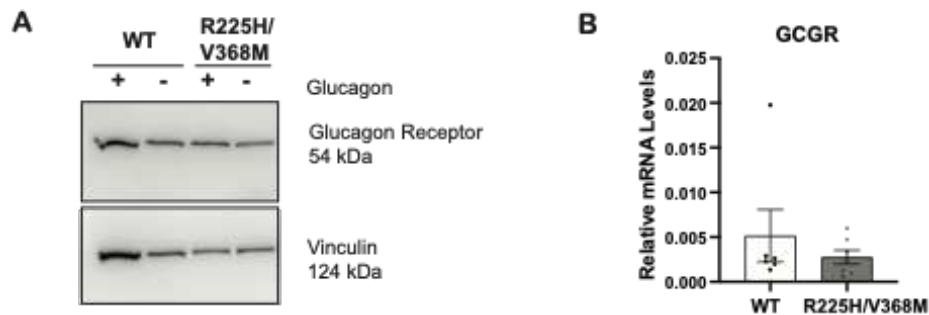
(A) Negative staining using an isotype control antibody. (B) FGF21 immunostaining of liver biopsy samples from *GCGR* mutation carriers at 1X magnification (top row) and 5X magnification (bottom row). From left to right: Case 1 (age 8 years), Mother (age 39 years), Case 2 early biopsy (age 27 years), Case 2 explanted liver (age 50 years). (C) FGF21 immunostaining of normal sections of liver, obtained from people undergoing liver

transplantation for distant hepatic adenomas. (D) FGF-21 immunostaining on liver tissue with underlying Metabolic dysfunction-associated steatotic liver disease (MASLD). (E) FGF-21 immunostaining on liver tissue from individuals undergoing liver transplantation for cirrhosis secondary to MASLD. All six individuals had concomitant hepatocellular carcinoma, but these biopsies were obtained from regions which did not harbour dysplasia or cancer. Brown staining = FGF-21, Blue staining = nuclei, Scale bar = 500 μ m. Patient details are included in Supplementary Table 2.

Supplementary Figure S3. Giemsa-band analysis of the wild type and mutant iPSC lines

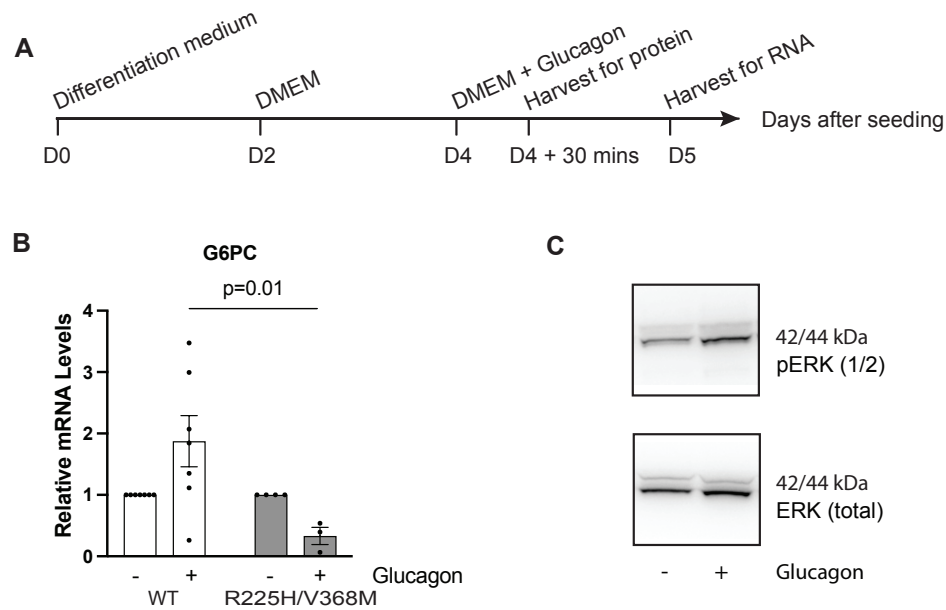


Supplementary Figure S4. Expression of the Glucagon Receptor in mature WT and mutant hepatocytes



(A) Expression of the Glucagon Receptor in mature WT and mutant hepatocytes, with/out glucagon stimulation, measured by Western Blot. Molecular weight = 54 kDa (B) Expression of the Glucagon Receptor in WT and mutant hepatocytes measured by qPCR, relative to the housekeeping gene HMBS. Mean $\pm$ SEM. N = 6-7 experiments.

Supplementary Figure S5. Quantifying the response to glucagon



(A) Protocol summary following day of seeding (Day 0). Cells are seeded in complete differentiation medium. After 48 hours, they are starved of glucagon in high-glucose DMEM, supplemented with non-essential amino acids, chemically defined lipid concentrated, glutamate and oncostatin M. After 48 hours, they are treated with 100 nM glucagon or vehicle (5% acetic acid) and harvested as indicated. (B) Expression of G6PC, a canonical GCGR target gene, relative to the housekeeping gene UBC, in response to 100 nM glucagon for 24 hours in WT (white bars) v mutant (grey bars). Results are normalised for the treated condition in each experiment. Mean $\pm$ SEM. N=3-7 (C) ERK phosphorylation in response to 30 minutes glucagon stimulation (100 nM). pERK, DMEM = Dulbecco's modified eagle medium

Supplementary Table S1. Genetic variants in Case 1 and Case 2 among the curated 93-gene panel (Categories A-C).

Category	Gene	HGVS _c	HGVS _p	rsID:alt allele	Genotypes				AF gnomAD SAS	AF highest	Population with highest AF (gnomAD, 1000Genomes)
					Case 1	Case 2	Mother of Case 1	Father of Case 1			
A	GCGR	ENST00000400723.3:c.1102G>A	Val368Met	rs771824180:A	1/1	1/1	1/1	0/1	0.00026	0.00026	gnomAD_SAS
	GCGR	ENST00000400723.3:c.674G>A	Arg225His	rs371217388:A	1/1	1/1	1/1	0/1	0.0018	0.0061	SAS
B	PNPLA3	ENST00000216180.3:c.444C>G	Ile148Met	rs738409:G	0/1	0/1	.	0/1	0.22	0.55	gnomAD_AMR
	PNPLA3	ENST00000216180.3:c.1300A>G	Lys434Glu	rs2294918:G	1/1	0/1	0/1	0/1	0.74	0.90	AFR
	GCKR	ENST00000264717.2:c.1337T>C	Leu446Pro	rs1260326:C	1/1	0/1	1/1	1/1	0.76	0.91	AFR
	HSD17B13	ENST00000328546.4:c.812+2dup	Splice region	rs72613567:TA	.	0/1	.	.	0.15	0.34	EAS
	TM6SF2	ENST00000389363.4:c.499G>A	Glu167Lys	rs58542926:T	.	0/1	.	.	0.090	0.11	SAS
C	APOB	ENST00000233242.1:c.728A>G	Gln243Arg	.	0/1	.	0/1	.	.	.	.
	RB1CC1	ENST00000025008.5:c.799C>G	Pro267Ala	.	0/1	.	0/1	0/1	.	.	.
	PNPLA2	ENST00000336615.4:c.756C>G	Asn252Lys	rs140201358:G	0/1	.	0/1	.	0.0069	0.014	EUR
	SREBF2	ENST00000361204.4:c.2786C>T	Ala929Val	rs200826207:T	0/1	.	.	0/1	0.0056	0.012	SAS

Footnotes. Category A: Rare coding variants (AF<1% in gnomAD SAS) for which both Case 1 and Case 2 were homozygous. **Category B:** Variants in genes containing common coding/splice region variants associated with liver fat and liver disease susceptibility(1). **Category C:** Other rare coding variants (AF<1% in gnomAD SAS) in Case 1 and/or Case 2.

The remaining genes in the 93-gene panel did not contain rare coding variants (AF<1% in gnomAD SAS) in Case 1 or Case 2 (not shown).

Variant annotation from Ensembl Variant Effect Predictor (VEP) v96 and v109 (GRCh37/hg19) with allele frequencies (AF) from gnomAD v2.1 exomes and 1000 Genomes.

HSD17B13 rs72613567: TA was previously associated with reduced risk of nonalcoholic steatohepatitis(2).

Genotypes displayed as 0/1 (heterozygous), 1/1 (homozygous alternate allele).

Human Genome Variation Society (HGVS) coding sequence (HGVSc) and protein sequence name (HGVSp) are shown.

Subpopulations in The Genome Aggregation Database (gnomAD): South Asian, SAS; AMR, Admixed American; EUR, non-Finnish European; AFR, African/African American; EAS, East Asian.

Supplementary Table S2. Characteristics of individuals providing control liver tissue

A – No chronic liver disease

Age (years)	Sex	BMI (kg/m ²)	Diabetes	Aetiology of Liver Disease
26	F	38.6	Yes	Hepatic adenoma
34	M	26.8	No	Hepatic adenoma
59	F	27.7	No	Hepatic adenoma
40	F	21.2	No	Hepatic adenoma
70	F	31.1	No	Hepatic adenoma

B – Early Metabolic Dysfunction-associated Steatotic Liver Disease (MASLD) without type 2 diabetes

Age (years)	Sex	BMI (kg/m ²)	Liver Biopsy Clinical Research Network Scoring System			
			Steatosis (0-3)	Ballooning (0-2)	Inflammation (0-2)	Fibrosis (Brunt)
57	M	23.6	1	0	1	1a
39	M	24.5	2	1	1	1a
51	M	26.8	2	1	0	0
28	M	28.4	2	1	0	0
49	M	29.1	1	0	1	1a
73	M	29.9	1	0	1	1c

C – Liver transplant for MASLD and Hepatocellular carcinoma (HCC)

Age (years)	Sex	BMI (kg/m ²)	Diabetes	Primary Aetiology of Liver Disease	Secondary aetiology of Liver Disease
59	M	42	Yes	MASLD	HCC
59	M	39	No	MASLD	HCC
58	M	35	Yes	MASLD	HCC
58	M	29	Yes	MASLD	HCC
54	M	39	Yes	MASLD	HCC
59	M	36	Yes	MASLD	HCC

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