Research Article
MASLD and Alcohol-Related Liver Diseases

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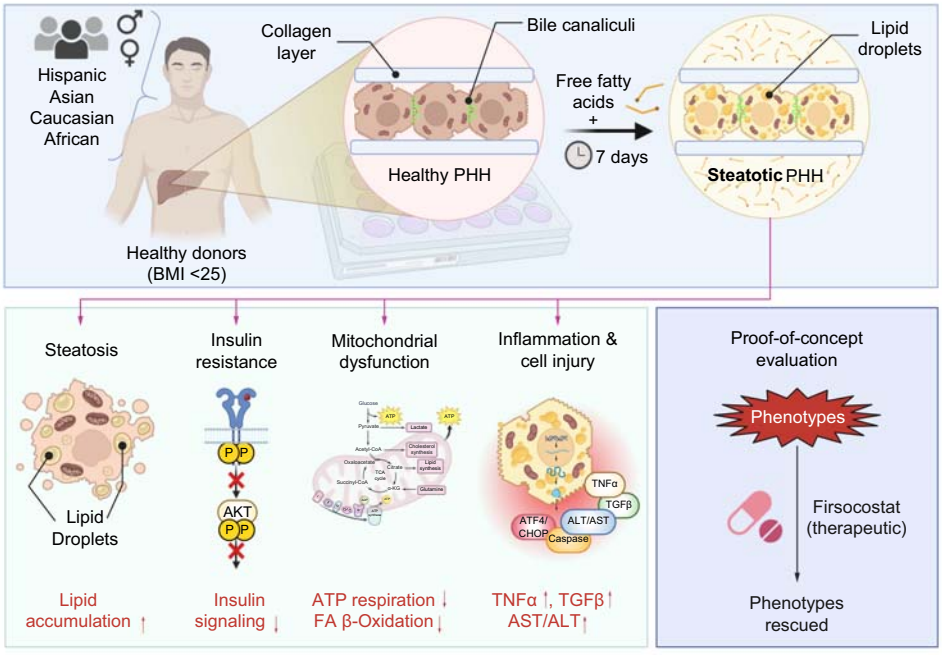
Graphical abstract



Highlights

- Increasing prevalence of MASLD warrants new cellular models for drug development.
- *In vitro* model of steatotic primary human hepatocytes recapitulates MASLD phenotypes.
- Firsocostat rescued steatosis and insulin resistance, providing proof of concept.

Impact and implications

Due to low drug efficacy and high toxicity, clinical treatment options for metabolic dysfunction-associated steatotic liver disease (MASLD) are currently limited. To facilitate earlier stop-go decisions in drug development, we have established a primary human steatotic hepatocyte *in vitro* model. As the model recapitulates clinically relevant MASLD characteristics at high phenotypic resolution, it can serve as a pre-screening platform and guide target identification and validation in MASLD therapy.

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Induction of steatosis in primary human hepatocytes recapitulates key pathophysiological aspects of metabolic dysfunction-associated steatotic liver disease

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Background & Aims: Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common cause of chronic liver disease. Owing to limited available treatment options, novel pre-clinical models for target selection and drug validation are warranted. We have established and extensively characterized a primary human steatotic hepatocyte *in vitro* model system that could guide the development of treatment strategies for MASLD.

Methods: Cryopreserved primary human hepatocytes from five donors varying in sex and ethnicity were cultured with free fatty acids in a 3D collagen sandwich for 7 days and the development of MASLD was followed by assessing classical hepatocellular functions. As proof of concept, the effects of the drug firsocostat (GS-0976) on *in vitro* MASLD phenotypes were evaluated.

Results: Incubation with free fatty acids induced steatosis, insulin resistance, mitochondrial dysfunction, inflammation, and alterations in prominent human gene signatures similar to patients with MASLD, indicating the recapitulation of human MASLD in this system. The application of firsocostat rescued clinically observed fatty liver disease pathologies, highlighting the ability of the *in vitro* system to test the efficacy and potentially characterize the mode of action of drug candidates.

Conclusions: Altogether, our human MASLD *in vitro* model system could guide the development and validation of novel targets and drugs for the treatment of MASLD.

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common liver disease with a prevalence of 25% in the general population.[1] Strongly associated with obesity, type 2 diabetes (T2D), and dyslipidemia, MASLD pathologies range from rather harmless hepatic steatosis (metabolic dysfunction-associated steatotic liver, MASL) to the more severe form of metabolic dysfunction-associated steatohepatitis (MASH), formerly referred to as non-alcoholic steatohepatitis (NASH). MASH is characterized by liver inflammation and fibrosis and can further develop into cirrhosis and hepatocellular carcinoma (HCC) – end-stage liver diseases for which liver transplantation remains the only cure.

Just recently, the FDA approved the thyroid hormone receptor agonist resmetirom for the treatment of MASH (clinical trial no. NCT03900429).[2,3] Whether this is sufficient to cure all stages of MASLD is yet to be seen, as no other drugs are currently available.[4] Treatment strategies usually center around lifestyle and dietary interventions; however, patient compliance is rather low and thus the disease can progress to cirrhosis, HCC, portal hypertension, encephalopathy, and liver failure.[5] As the forecasted number of patients is set to rise to two billion worldwide in the next 5-10 years,[6] the management of MASLD and its associated complications will cost billions of dollars, representing an immense socioeconomic burden. Moreover, MASLD is considered a high-risk factor for metabolic comorbidities strongly associated with the progression of cardiovascular diseases,[7] hypercholesterolemia, and severity of T2D,[8] thus restricting MASLD progression should also have benefits on other metabolic diseases. Therefore, there is a strong need for reliable and effective drugs to treat MASLD. However, many recent attempts to develop new MASH pharmacotherapies have failed in phase IIb and III clinical trials due to toxicity and low efficacy, questioning target selection.[9]

The disease progression to MASH is complex and involves triglyceride (TG) accumulation, insulin resistance, mitochondrial dysfunction, and inflammation,[10] including activation of Kupffer

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cells and hepatic stellate cells. However, dietary and environmental factors, genetic predisposition,[11] and crosstalk between the liver and other organs, such as adipose tissue, also contribute to the development of MASLD.[12] Due to the complexity of the pathogenic mechanism, the discovery of efficacious cellular targets and pharmaceutical drugs has been extremely complicated. The translational capability of MASLD-mimicking dietary studies in mice appears to be limited,[13] calling for additional screening strategies and platforms for more effective drug screening prior to human translation.

To overcome this gap, human hepatic cell lines, hepatocyte-like cells, and primary human hepatocytes (PHHs) have been developed as *in vitro* cell culture models.[14] Human cell lines are widely used in academic research and by the pharmaceutical industry thanks to their capacity for long-term culture. However, due to their immortalization they do not maintain metabolic and cell type-specific (hepatocyte-like) functions.[15] This makes them less predictable and usable for drug screening in MASLD. In contrast, primary hepatocytes, which are directly isolated from the liver, have the capacity to maintain their glucose and lipid metabolic profiles. Culturing primary mouse and human hepatocytes in a 3D collagen sandwich system ensures their bipolar hepatocyte-like morphology[16] and thus their metabolic functions in glucose and lipid homeostasis.[17–19] Although such 3D culture models are used for general drug toxicology studies on liver function,[20] they have not been applied to a MASLD setup. In addition, no existing MASLD *in vitro* system systematically investigates to what extent such models mimic all aspects of human MASLD pathologies.

In this study, we present a robust 3D human steatotic hepatocyte *in vitro* system that recapitulates major hallmarks of MASLD, including steatosis, insulin resistance, mitochondrial dysfunction, and inflammation. Using PHH cultures from five donors we show that their transcriptome signature is similar to that of patients with MASLD. Given its reversibility and a positive proof-of-concept evaluation of the MASLD drug firsocostat (GS-0976, currently in a phase IIb clinical trial; no. NCT03449446), our system provides a screening platform that can be used for validation studies and guide stop-go decisions in early-phase MASLD therapeutic development.

Materials and methods

Donor information

Cryopreserved PHHs were purchased from Lonza Group Ltd (Valais, Switzerland) or BioIVT (Westbury, U.S.A; West Sussex, U.K); information on single-donor hepatocytes is indicated in Table S1. Usage of PHHs from individual donors per experiment is indicated in Table S2.

Statistical analysis

Statistical analysis was performed in GraphPad Prism (v9.5.1) using Student's *t* test (two-tailed, unpaired), or one-way or two-way ANOVA. The *p* values were corrected with multiple comparisons via the two-stage step-up method of Benjamini, Krieger, and Yekutieli. $p < 0.05$ was considered statistically significant. Data represent group means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, calculated by unpaired two-tailed *t* test or two-way ANOVA.

More information can be found in the supplementary CTAT table and supplementary information file.

Results

Treatment with free fatty acids induces steatosis and lipotoxicity in PHHs

Induction of steatosis in hepatocytes is usually achieved by incubating these cells with relatively high amounts of palmitic acid (PA) and oleic acid (OA), the most abundant free fatty acids (FFAs) in the human diet.[21] This treatment is known to induce steatosis but also cytotoxicity and cell death in primary cultures of rat and human hepatocytes.[21] However, human MASLD development starts by accumulation of neutral lipids and TG, stored in lipid droplets (LD), without causing cell death of hepatocytes in early stages.[22] To better mimic the human disease condition, we established an *in vitro* system that develops steatosis while maintaining cell viability. We cultured PHHs from a male Hispanic donor, with normal body mass index, no alcohol consumption, and no diabetes (Table S1). The cells were cultured in a 3D collagen sandwich system, known to be essential for hepatocellular polarity and liver-like metabolic functions.[17] After attachment and sandwich formation, the cells were incubated with a mixture of PA and OA at 1:5 ratio at different concentrations of FFA of 150, 300, and 600 μM or BSA alone for 3 and 7 days (Fig. 1A) and their LD formation and TG accumulation was assessed over time. Confocal imaging and quantification of the size and occupancy of LD stained with boron-dipyrromethene (BODIPY) showed progressive steatosis induction in a time-and dose-dependent manner (Fig. 1B,C). Consistent with the formation of LD, TG accumulation also increased (Fig. 1D). To evaluate the effect of FFA mixtures on cytotoxicity, we measured cell viability and caspase-3 activity in PHHs and observed an activation of apoptosis only with high FFA concentrations and lipopolysaccharide (Fig. 1E,F), but not with the low-concentration FFA mixture of 25 μM PA and 125 μM OA. Importantly, culturing PHHs for 7 days did not induce hepatocellular de-differentiation, as albumin release was maintained (Fig. S1A) and no changes in the expression of de-differentiation markers were observed (Fig. S1B,C). These data indicate that treatment with low concentrations of FFA recapitulates hepatic steatosis without increasing cytotoxicity and hepatic de-differentiation.

Steatotic PHHs recapitulate insulin resistance leading to sustained hepatic glucose production

The progression of MASL to severe disease stages is characterized by induction of insulin resistance, mitochondrial dysfunction, and inflammation.[23,24] However, most *in vitro* models do not consider these different hallmarks of the disease. To study to what extent our *in vitro* system recapitulates different MASLD pathologies, we first evaluated whether the incubation of PHHs with low concentrations of FFAs for 7 days results in insulin resistance. Indeed, induction of steatosis was associated with an approx. 60% decrease in insulin-induced phosphorylation of the insulin receptor (IR) and protein kinase B (AKT) (Fig. 2A–D). Consequently, insulin failed to suppress hepatic glucose production from steatotic PHHs measured by glucose release into the medium (Fig. 2E). These data indicate that steatotic PHHs indeed recapitulate insulin resistance with the consequence being sustained hepatic glucose production.

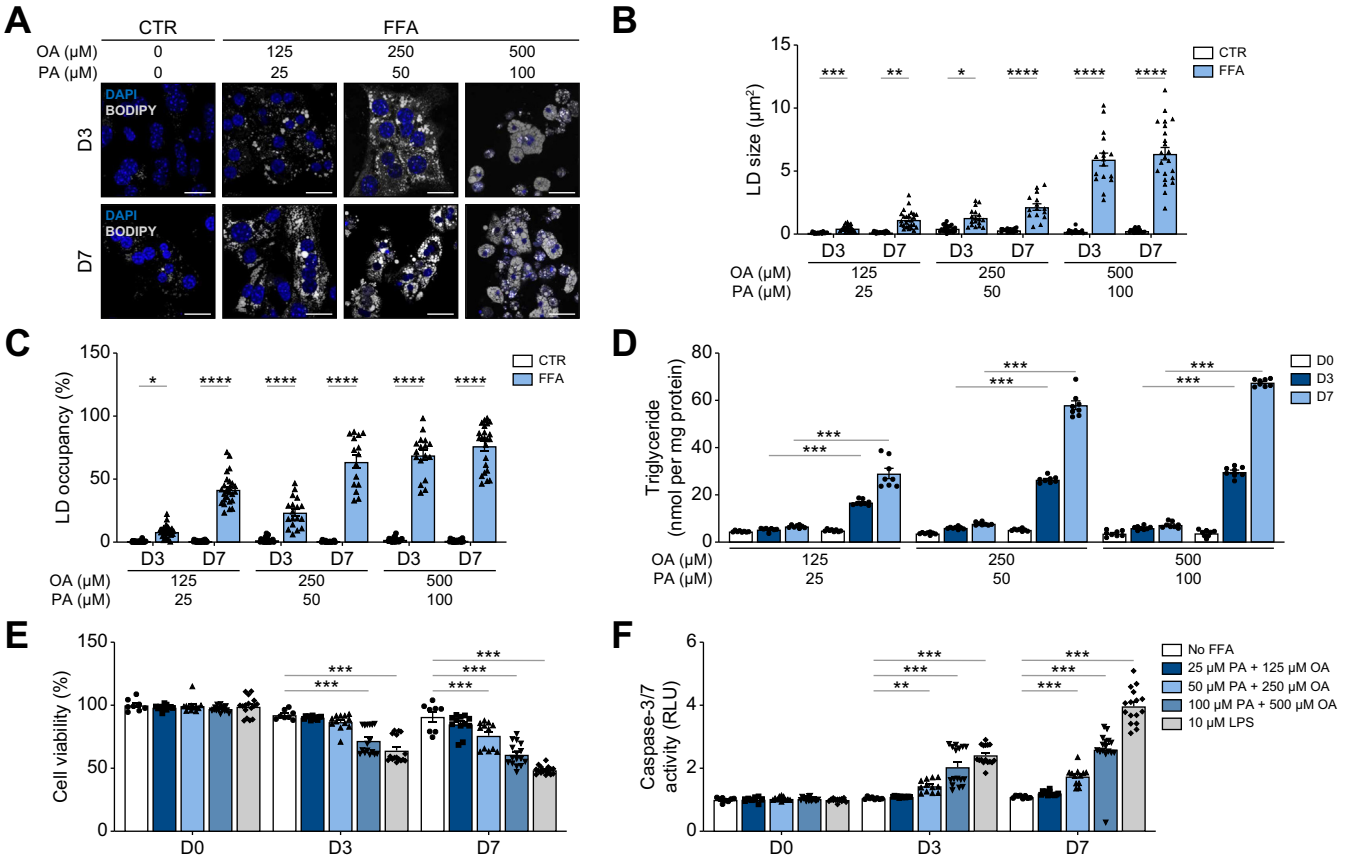

Fig. 1. Free fatty acids induce steatosis and lipotoxicity in PHHs. PHHs were treated for up to 7 days with BSA (CTR) or FFA (steatotic PHHs). (A) Representative images of LDs stained with BODIPY (grey) and DAPI (nuclei, blue) Scale bars, 20 μm. Quantification of LD size (B) and occupancy (C) from images in (A). (D) Triglyceride content, (E) cell viability, and (F) caspase-3/7 activity. Data are presented as mean ± SEM. *p <0.05; **p <0.01; ***p <0.001 (two-way ANOVA). FFA, free fatty acid; LD, lipid droplet; PHHs, primary human hepatocytes.

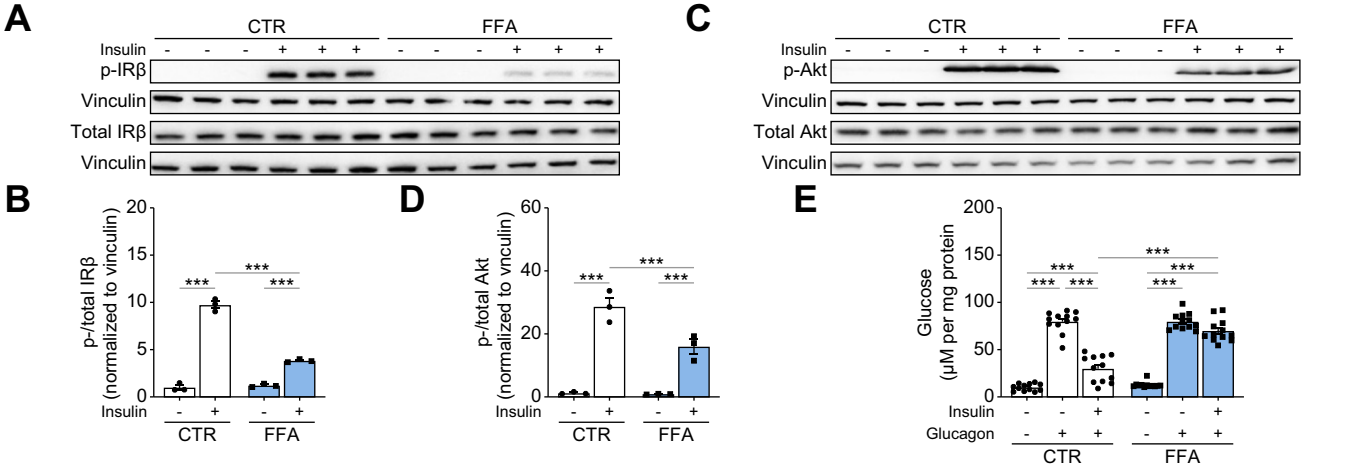

Fig. 2. Steatotic PHHs recapitulate insulin resistance leading to sustained hepatic glucose production. (A-D) Western blotting of CTR and FFA-treated (steatotic) PHHs, treated at day 7 for 10 min with 10 nM insulin or with equivalent amount of DMSO. (A) Representative immunoblots and (B) quantification of phosphorylated and total IR-β. (C) Representative immunoblots and (D) quantification of phosphorylated and total Akt. Individual bands indicate technical replicates; vinculin, loading control. (E) Glucose release from PHHs at day 7 treated for 10 min with 10 nM glucagon and 10 nM insulin as indicated. Data are presented as mean ± SEM. ***p <0.001 (two-way ANOVA). FFA, free fatty acid; IR-β, insulin receptor beta subunit; PHHs, primary human hepatocytes.

Steatotic PHHs show mitochondrial dysfunction and induction of pro-inflammatory and fibrotic states

As lipid accumulation and insulin resistance in the liver are associated with mitochondrial dysfunction,[25] we next evaluated the oxygen consumption rate (OCR) in steatotic PHHs *vs.* BSA-treated controls. Interestingly, steatotic hepatocytes exhibited a reduction in OCR, basal respiration, and ATP production (Fig. 3A,B) by approx. 30 to 35%. Moreover, fatty acid beta-oxidation (FAO) was reduced by 44% (basal FAO) and 42% (maximal FAO) (Fig. 3C,D), indicating impaired mitochondrial function. This coincided with a fragmentation of the mitochondrial network, as assessed by staining of the mitochondrial outer membrane with TOM20 (Fig. 3E–G). This was consistent with commonly found mitochondrial abnormalities in mouse and human cases of MASLD.[26] Interestingly, mitochondria have been shown to adapt and increase oxidative capacity in obese patients with simple hepatic steatosis. However, with more advanced stages of MASLD, hepatic mitochondrial function declines,[27] suggesting that our system recapitulates a more severe stage of MASLD.

The progression of MASH is driven by the occurrence of inflammation, which is a key clinical hallmark of severe fatty liver disease. Although hepatic stellate and Kupffer cells are the main drivers of liver inflammation,[28] their activation and recruitment also depend on the inflammatory state of hepatocytes. Interestingly, FFA treatment for 7 days increased gene expression of pro-inflammatory cytokines in steatotic PHHs (Fig. 3H) and enhanced TNF-α (Fig. 3I) and TGF-β (Fig. 3J) secretion into the

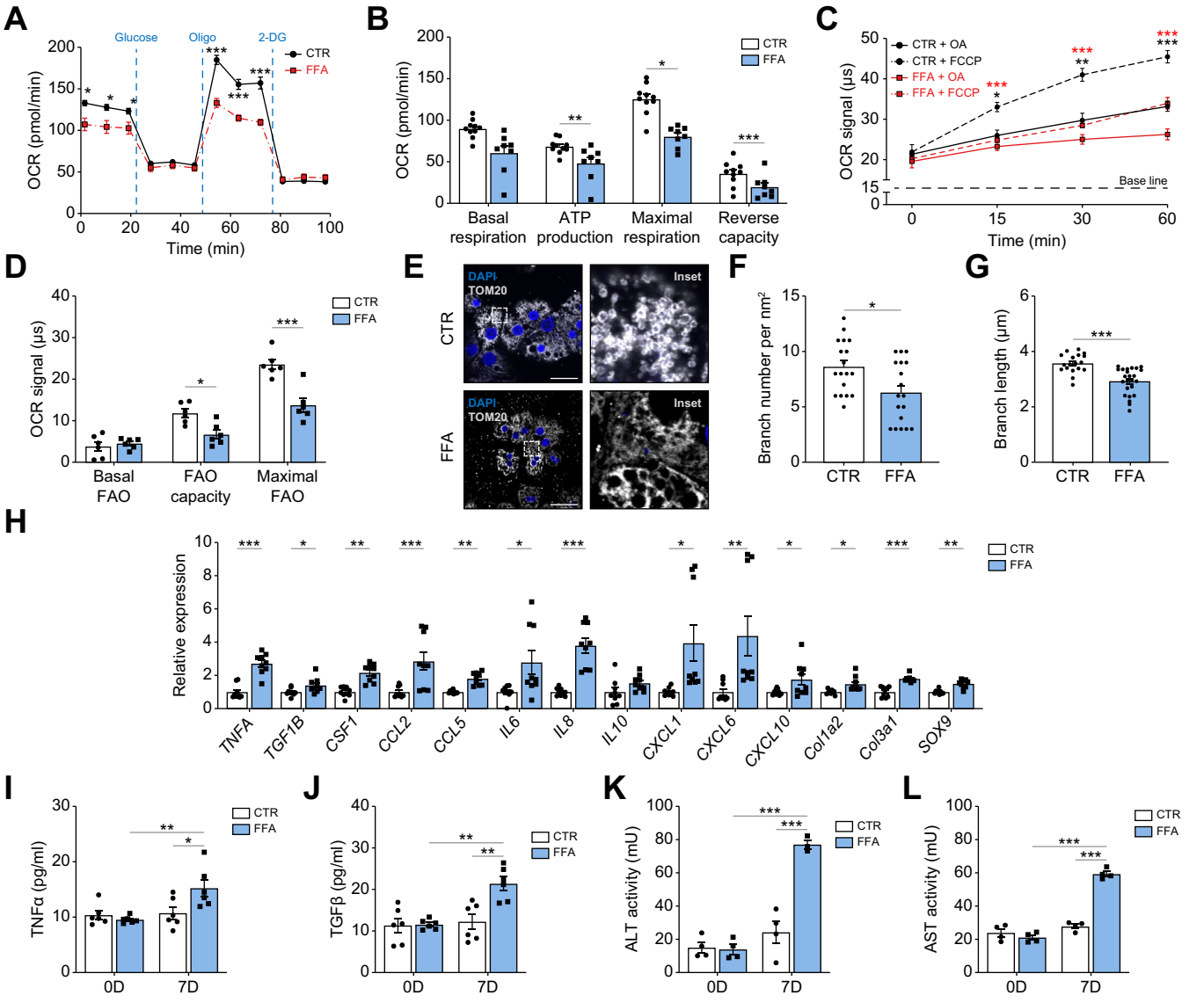

Fig. 3. Steatotic PHHs show mitochondrial dysfunction and induction of pro-inflammatory and fibrotic states. (A) OCR in CTR and FFA-treated (steatotic) PHHs at day 7. (B) Individual OCR components. (C) OCR from the FAO triggered by BSA, BSA with FCCP, OA or OA with FCCP. (D) individual FAO components. (E) Representative images of mitochondria at day 7. TOM20: mitochondrial outer membrane; DAPI, nuclei; scale bars, 20 µm. (F) Mitochondrial branch number per cell area; (G) mitochondrial branch length. (H) mRNA expression of pro-inflammatory cytokines and fibrosis markers; (I-L) TNF-α, TGB-β, ALT, and AST release. Data are presented as mean ± SEM. *p <0.05; **p <0.01; ***p <0.001 (unpaired two-tailed t-test for A-H; two-way ANOVA for I-L). ALT, alanine aminotransferase; AST, aspartate aminotransferase; FAO, fatty acid oxidation; FFA, free fatty acid; OA, oleic acid; OCR, oxygen consumption rate; PHHs, primary human hepatocytes. (This figure appears in color on the web.)

medium, even in the absence of immune cells. Pro-inflammatory cytokines caused an intrinsic activation of fibrotic gene expression of pro-collagens and their transcription factor *SOX9* (Fig. 3H), supporting an advanced inflammatory state of the PHHs. Importantly, this was not caused by hepatic stellate cell contamination of our cell culture system. As determined by RNA-Seq, stellate cell-specific genes were either expressed at very low levels (*ACTA2* and *LRAT*) or could not be detected at all (*VIM* and *DCN*) in both control and FFA-treated cells relative to hepatocyte marker genes (*ALB* and *TF*) (Fig. S2). Furthermore, none of these genes were upregulated in FFA-treated compared to control cells, indicating that the increased pro-collagen gene expression could be attributed to hepatocytes, rather than hepatic stellate cells. In fact, we observed an increased release of alanine aminotransferase and aspartate aminotransferase into the medium from the PHHs (Fig. 3K,L), demonstrating detrimental effects of long-term lipid exposure on hepatocellular health. To elucidate the underlying molecular mechanisms of the observed MASLD phenotypes, we analyzed cell stress and injury pathways. Interestingly, expression of endoplasmic reticulum (ER) stress-related marker genes and proteins (Fig. S3A-C), cleavage of caspase-3 (Fig. S3D,E), and reactive oxygen species (ROS) production (Fig. S3F) were all elevated in steatotic PHHs. This strongly suggests that induction of cell stress and injury upon FFA treatment contributes to MASLD development. Altogether, these data demonstrate that the steatotic PHH *in vitro* system recapitulates major hallmarks of MASLD and can be used to evaluate drugs targeting specific features of the human disease.

Development of fatty liver pathologies is reversible, predictive, and reproducible

Essential for an *in vitro* system for effective drug and target validation are 1) reversibility, 2) predictability, and 3) reproducibility of the observed disease phenotypes. For this, we first tested whether steatosis and insulin resistance could be rescued by simply removing FFAs from the medium. Hence, PHHs were cultured for 3 days with 150 μM FFAs followed by incubation with FFA-free medium for an additional 4 days *vs*. full treatment with FFAs for 7 days. As expected, exposure to FFAs induced LD accumulation and insulin resistance already after 3 days, which could be reduced by changing the medium to BSA alone (Fig. S4). Interestingly, although the removal of FFAs only partially reduced LD size and occupancy (Fig. S4A-C), it completely rescued the effect of FFAs on insulin resistance (Fig. S4D-H), suggesting that lipid accumulation may not directly cause insulin resistance and lipotoxicity.

To further examine its potential utility as a drug validation platform, we tested whether findings from clinical and rodent studies can be phenocopied within the human MASLD *in vitro* system. Drugs against MASLD are designed to either block excessive lipid deposition or decrease hepatic inflammation and fibrosis.[29,30] Thus, a potent drug candidate is firsocostat, an inhibitor of acetyl-CoA carboxylases (ACC) 1 and 2. These rate-limiting enzymes cause lipid accumulation by promoting *de novo* lipogenesis (ACC1) and by blocking fatty acid beta-oxidation (ACC2).[31] Indeed, inhibition of ACC1/2 by firsocostat reduces hepatic steatosis in humans[32] and improves insulin resistance in animal models.[33] Treating PHHs, where steatosis was induced for 3 days, with medium containing FFA plus 10 μM firsocostat for an additional 4 days remarkably reduced the area and size of LDs (Fig. 4A–C). In addition, TG levels were significantly reduced (Fig. 4D), in agreement with the human patient data. As firsocostat was shown to improve insulin sensitivity in rodents, but has not been evaluated in humans, we assessed the ability of steatotic PHHs to respond to insulin stimulation after firsocostat treatment by measuring phosphorylation of the IR. Interestingly, exposure to firsocostat for 4 days reduced insulin resistance by approx. 50% (Fig. 4E,F), indicating a positive effect of firsocostat on insulin sensitivity in steatotic PHHs.

As our PHH culture recapitulates additional features of MASLD development, we tested whether firsocostat influences mitochondrial function, FAO, and inflammation. Interestingly, treatment with firsocostat strongly enhanced mitochondrial OCR (Fig. 4G,H), had no effect on basal beta-oxidation, but restored maximal FCCP-induced oxidation (Fig. 4I,J) and partially improved pro-inflammatory markers, especially *TGF-β* and *CCL2* (Fig. 4K).

As there are genetic variations between patients with MASLD, it is essential to establish an *in vitro* system that can reproduce the different disease phenotypes independently of ethnicity and sex.[34] Thus, we expanded our analysis to four additional hepatocyte donors, including Asian, Caucasian, and African American ethnicities, coming from two female and two male donors (Table S1). All were selected to have normal body mass index, no diabetes, and no or only social alcohol consumption, similar to the male Hispanic donor used to establish the MASLD *in vitro* system (Fig. 1-4). Thus, we tested the MASLD phenotypes side-by-side in those donors with or without firsocostat and indeed observed a robust induction of LD formation (Fig. 5A–C), TG accumulation (Fig. 5D), and insulin resistance (Fig. 5E,F) when treated with a 150 μM FFA mixture for 7 days. Interestingly, a reduction in OCR in response to FFA treatment was only observed in PHHs from male, but not female donors, suggesting a sex-dependent difference in lipid-induced mitochondrial dysfunction (Fig. S5A-D). Indeed, steatotic PHHs from female donors exhibited no fragmentation of the mitochondria, in agreement with unchanged OCR (Fig. S5E-J). Importantly, firsocostat was able to improve both steatosis and insulin sensitivity in all donors, albeit to different degrees. As expected, firsocostat only restored mitochondrial respiration in male donors (Fig. 5C,D). To exclude the possible contribution of technical variations to the observed donor-to-donor heterogenetic responses, we estimated the standard deviations between independently measured induction of steatosis, TG accumulation, and insulin resistance and their improvement upon firsocostat treatment of PHHs from the Hispanic donor. Interestingly, technical variations fluctuated only slightly but not significantly between different experiments (Fig. S6) and thus could not contribute to the variations between donors. Altogether, these data provide a proof of concept of the steatotic PHH *in vitro* system to recapitulate clinical findings of MASLD drugs and to evaluate additional parameters associated with MASLD pathologies.

Steatotic PHH *in vitro* system recapitulates gene signatures associated with MASLD/pre-MASH from patient samples

To elucidate to what extent the steatotic PHH *in vitro* system reproduces known gene signatures of patients with MASLD, we

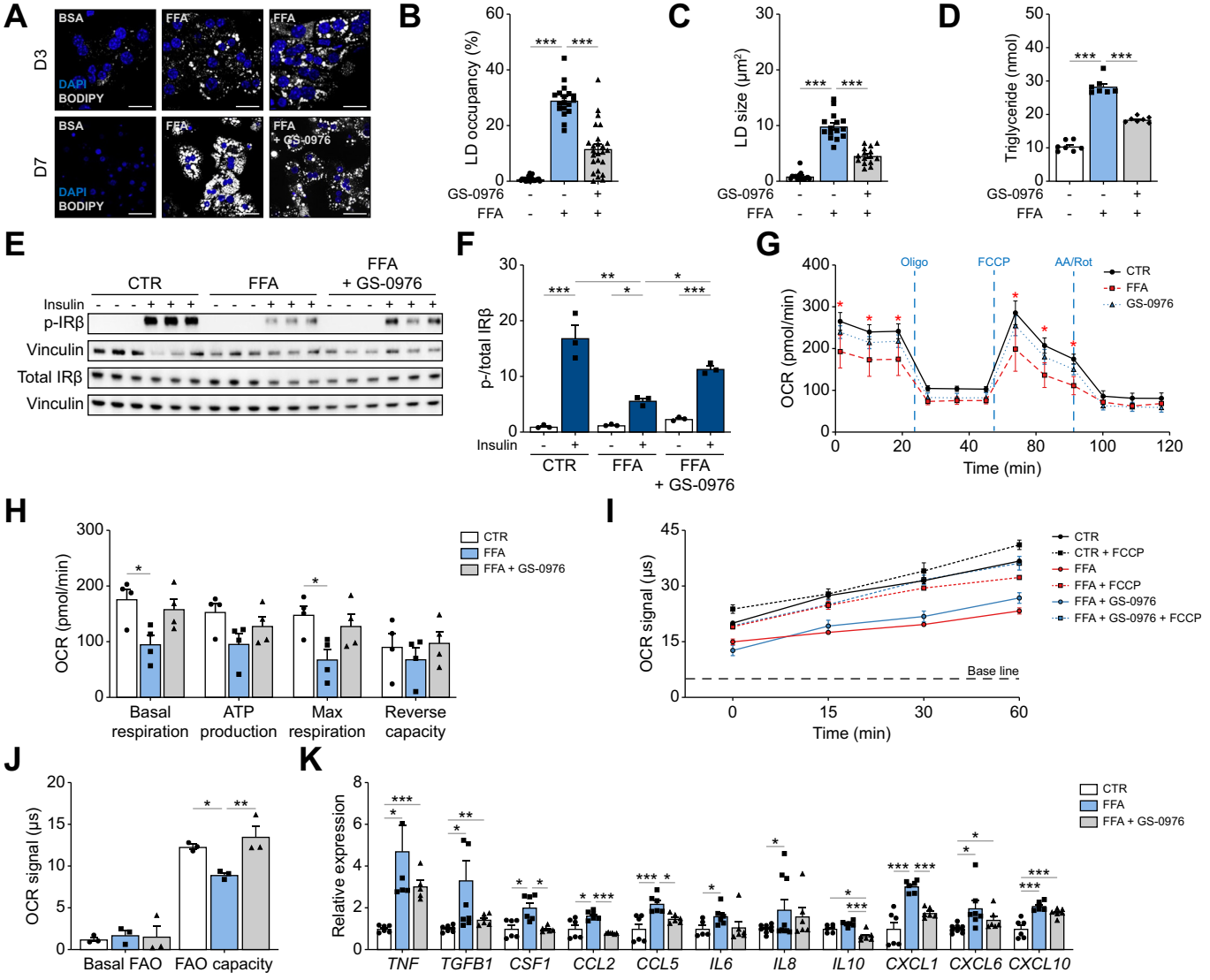

Fig. 4. Fatty liver phenotype is reversible, predictive, and reproducible. PHHs were treated with BSA (CTR), FFAs for 7 days, or FFAs for 3 days followed by FFAs + 10 μM GS-0976 (firsocostat) for another 4 days. (A) Representative images of LDs (BODIPY); DAPI, nuclei; scale bars, 20 μm. (B) LD occupancy and (C) LD size. (D) Triglyceride content. (E) Representative immunoblots and (F) quantification of phosphorylated and total IR-β. (G) OCR and (H) individual OCR components. (I) OCR of hepatic FAO upon stimulation with exogenous OA; (J) individual OCR FAO components. (K) mRNA expression of pro-inflammatory cytokines. Data are presented as mean ± SEM. *p <0.05; **p <0.01; ***p <0.001 (two-way ANOVA). FAO, fatty acid oxidation; FFAs, free fatty acids; IR-β, insulin receptor beta subunit; LD, lipid droplet; OA, oleic acid; OCR, oxygen consumption rate; PHHs, primary human hepatocytes. (This figure appears in color on the web.)

conducted RNA sequencing (RNA-Seq) of PHH samples from five donors treated side-by-side with FFA *vs*. BSA control over a 7-day period. The principal component analysis (PCA) revealed a clear separation between samples from donors of different sexes, as well as different ethnicities (Fig. S7). However, we still observed a separation between BSA- and FFA-treated samples within the cluster of single donors, indicating the effect of FFA was reflected also on a transcriptional level (Fig. S7). We found 405 transcripts to be differentially expressed in PHHs, where the LD coating protein perilipin-2 (*PLIN2*) was most highly up-regulated,[35] confirming massive lipid accumulation (Fig. 6A). Interestingly, in combination with clinical parameters, the PLIN2 protein in monocytes is a novel liquid biopsy biomarker for MASH, superior to clinically used indexes of MASLD.[36] Pathway enrichment analysis of the 405 transcripts identified multiple pathways linked to fatty acid metabolism and signaling (Fig. 6B), consistent with transcriptome data from liver samples of patients with MASLD.[37]

As genetic predisposition is a strong driver of MASLD, we used RNA-Seq to assess the occurrence of genetic variants potentially driving the fatty liver development. Among the detected risk alleles *GCKR*, *TM6SF2*, *PNPLA3*, and *SERPINA1* (Table S3), we observed a clear heterogeneity among the five donors. These data indicate that the induction of MASLD is independent of risk variants.

To test if the observed transcriptional changes recapitulate changes in patients with MASLD, we aligned 405 differentially expressed genes (DEGs) from our dataset against 870 MASLD liver samples from expression studies available at the GENEVESTIGATOR expression database(38). Strikingly, we observed

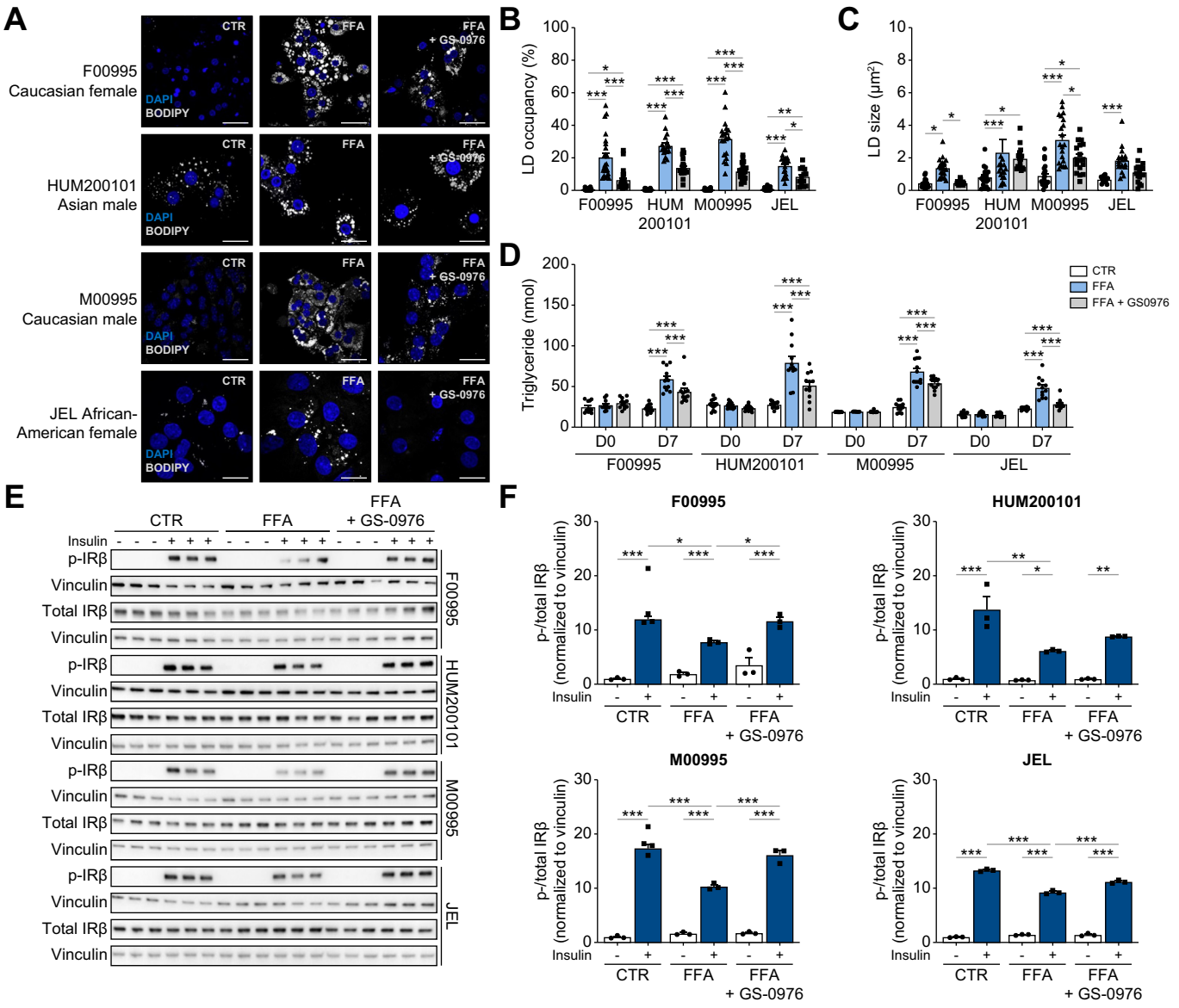

Fig. 5. Steatosis and insulin resistance are recapitulated in PHHs from different donors. Independent PHH cultures from four additional donors were treated with BSA (CTR), FFAs for 7 days, or FFAs for 3 days followed by FFAs + 10 µM GS-0976 (firsocostat) for another 4 days. (A) Representative images of LDs (BODIPY) in single-donor PHH; DAPI, nuclei; scale bars, 20 µm. (B) LD occupancy and (C) LD size. (D) Triglyceride content. (E) Representative immunoblots and (F) quantification of phosphorylated and total IR-β. Data are presented as mean ± SEM. *p <0.05; **p <0.01; ***p <0.001 (two-way ANOVA). FFA, free fatty acid; IR-β, insulin receptor beta subunit; LD, lipid droplet; OA, oleic acid; OCR, oxygen consumption rate; PHHs, primary human hepatocytes. (This figure appears in color on the web.)

a high similarity of the steatotic PHHs with many MASLD studies, with a Pearson correlation starting at 0.797 (Fig. 6C and Table S4), indicating a profound similarity of our PHH system to the transcriptional profiles of patients with MASLD. To verify expression changes related to fatty acid metabolism, we assessed individual gene alterations using qPCR, and indeed observed a strong increase of *de novo* lipogenesis (Fig. 6D).

Moreover, the principal component analysis clustered the steatotic PHHs with MASLD transcriptomes (Fig. S7B). Interestingly, our unbiased GENEVESTIGATOR analysis also indicated some similarities of the PHH gene signatures to human MASH studies. By comparing the 405 DEGs to MASH transcriptomes, we identified 41 genes (Fig. S7C), with many associated with extracellular matrix regulation (*CDHR2, TM4SF5*), epithelial-mesenchymal transition (*PHLDB2, FUT6, CHI3L1*), or cancer (*TM4SF5, FUT6,* B3GNT3*, SEMA4B*) (Table S5). Interestingly, we also detected *SLC17A4*, a novel thyroid hormone transporter[3,38] amongst the commonly altered genes. Given the newly approved MASH drug Rezdiffra (resmetirom),[2,3] these data further support the PHHs as a potential tool for MASH drug development.

To allow for an accurate prediction of expression changes related to impaired insulin sensitivity and elevated lipid storage in steatotic PHHs, we applied a machine learning approach, the LASSO regression. The 405 DEGs were tested in 1,000 random prediction models in relation to these two phenotypes. A model was designated successful if the estimated phenotype values

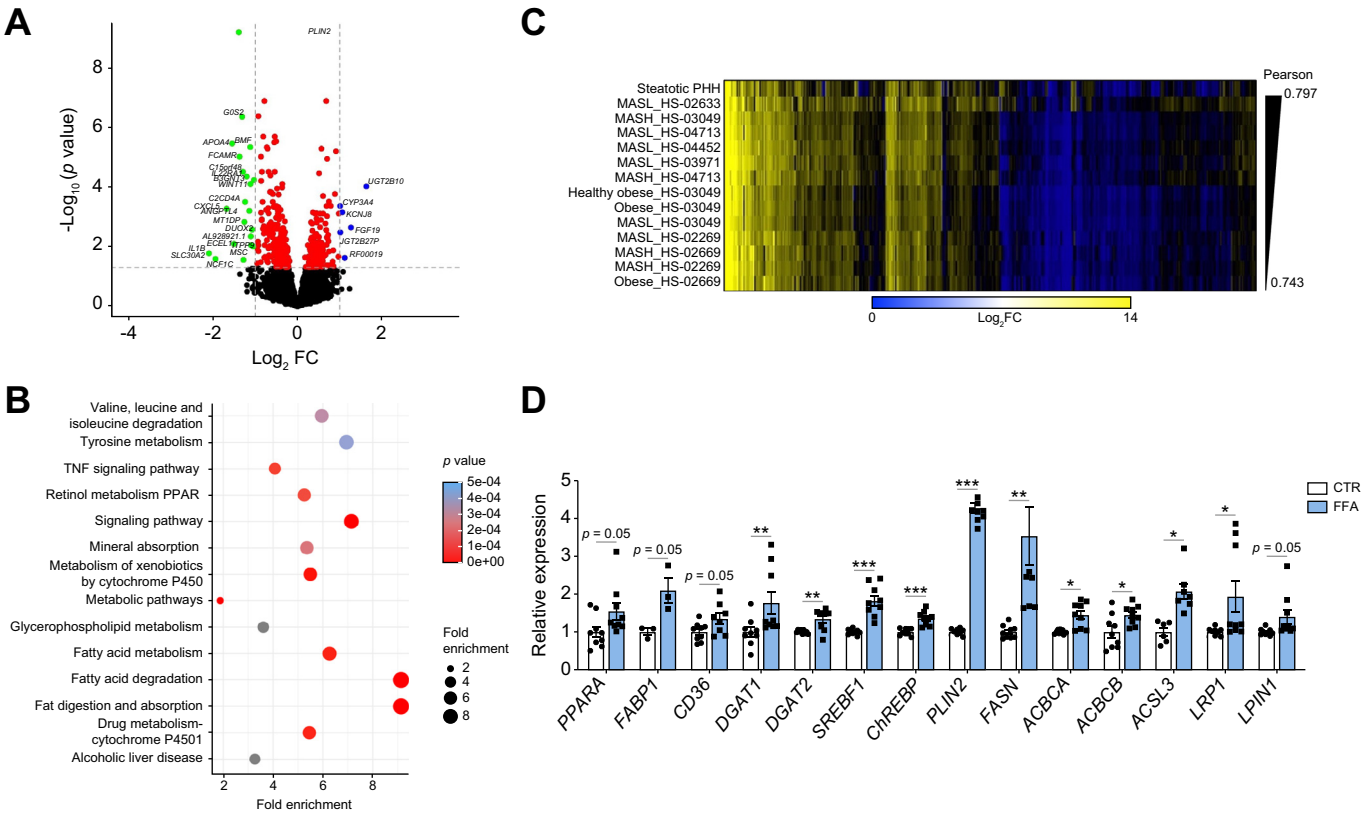

Fig. 6. Transcriptome and pathway analyses of steatotic PHHs reveals similarities to human MASLD/MASH studies. (A) Volcano plot with 405 significantly DEGs (green, up-regulated; blue, down-regulated; Log2FC> 1) in FFA-treated (steatotic) PHHs at day 7. (B) Corresponding pathway enrichment analysis. (C) Heatmap of 405 DEGs from (A) compared with those from MASLD/MASH patient livers in GENEVESTIGATOR database; data were sorted by similarities based on Pearson correlation (yellow, up-regulated; blue, down-regulated). (D) Relative gene expression analysis of *de novo* lipogenesis genes from CTR and FFA-treated PHHs from the Hispanic male donor. Data are presented as mean ± SEM. **p* <0.05; ***p* <0.01; ****p* <0.001 (unpaired two-tailed t-test). DEGs, differentially expressed genes; FFA, free fatty acid; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; PHHs, primary human hepatocytes. (This figure appears in color on the web.)

were less than 10% different from the measured ones in at least 50% of the samples. LASSO regression identified seven models for the effect on insulin sensitivity and nine for increased TG concentration. The ranking of the genes to be predictive of changes in phosphorylation of the IR and TG content is based on their occurrence in successful models. In total, 60 genes were estimated to be predictive of decreased insulin sensitivity (Fig. S8A) and 103 genes of elevated TG content (Fig. S8B). Interestingly, 39 genes were among successful models for both phenotypes and focusing on those that are detected in at least 50% of the models are nine candidates, which are all well established in MASLD (Fig. S8C). Also, the lists of genes exclusively predicting insulin sensitivity and TG content contain genes known to participate in fatty liver development. However, there are also genes that may be novel prediction markers (*DEFB1, TCIRG1, TMEM37*, and *TMEM45A*) and involved in MASH progression.

Discussion

Here, we present an *in vitro* 3D culture system of primary human steatotic hepatocytes that recapitulates clinically relevant characteristics of MASLD at high phenotypic resolution, including parameters of insulin resistance, mitochondrial dysfunction, and increased inflammation. Expression profiling and pathway analyses confirmed the resemblance of transcriptional changes to those in patients at different stages of MASLD, supporting the robust FFA-induced alterations on the gene level. Furthermore, LASSO regression identified both well-known and novel prediction marker genes for impaired insulin sensitivity and increased lipid storage.

The tremendous increase in numbers of patients with fatty liver diseases, inflammation, and associated metabolic diseases poses an unmet demand for new therapeutic approaches. Unfortunately, there is currently no drug approved for this indication, mainly because of the lack of efficacy, occurring toxicity, or limited usability for the different clinical aspects of MASLD. This is primarily due to the use of drug screening approaches that are incapable of phenocopying complex hepatic functions under disease development. Most of the normally used screening models involve short-term cultures (12-48 h) of hepatic cell lines or primary cells with excess FFA concentrations (up to 600 μM).[39] However, such high concentrations exceed physiological levels and thereby develop artificial cytotoxicity as well as inappropriate drug responses.[40] In addition, normal 2D cultures of hepatocytes lose their *in vivo* identity within 24 h and undergo epithelial-mesenchymal transition, thus changing their metabolic program away from a metabolically specialized cell.[41] In contrast, cultures of primary mouse and human hepatocytes in 3D collagen sandwiches maintain hepatocellular polarity and thus

liver-like metabolic functions.[17–19] Combining this with low concentrations of FFA (150 μM) given over longer periods of time (up to 7 days) to induce steatosis allows for an *in vitro* MASLD model system with low intrinsic toxicity and high hepatocellular functionality.

As our system is purely based on hepatocytes, complete MASH development cannot be mimicked due to the lack of multicellular crosstalk with intrahepatic immune cells. Nevertheless, manifestation of liver steatosis can exacerbate hepatic inflammation, leading to the release of pro-fibrotic and pro-inflammatory cytokines.[42] Interestingly, we observe an increase in pro-inflammatory gene expression and an induction of TNF-α and TGF-β secretion, emphasizing an intrinsic activation of the inflammatory program towards MASH transition. This was associated with the release of alanine aminotransferase and aspartate aminotransferase and mitochondrial dysfunction, further strengthening the progression of the PHHs to a pre-MASH state. Having these well-known MASLD biomarkers in place, this screening platform can also be used for lipotoxicity testing of novel drugs in a human setting.

The use of different donors allowed us to define the most robust phenotypes of MASLD. Despite the differences in sex and ethnicity, the induction of steatosis and insulin resistance are conserved amongst all donors and represent the most robust readouts for drug efficacy. This system also allows for predictions of potential differences in patients' long-term responses to drugs, which can be followed up and would have been overlooked when using pooled hepatocytes of multiple donors, which is common practice in the pharmaceutical industry.[39] Interestingly, we observed that none of the female donors showed FFA-induced mitochondrial dysfunction, suggesting an effect of sex on mitochondrial sensitivity to FFA. This agrees with patient data, where the susceptibility for developing MASLD is lower in females than in males.[43,44] However, due to low numbers of donors used here, these are only individual observations. Using cells from higher numbers of donors of similar ethnicity, sex, and age would help to draw stronger conclusions on individual genetic-based mechanisms on donor responses to treatments. In addition, employing hepatocytes differentiated from induced pluripotent stem cells from individual patients grown in spheroids[39] could be used to develop strategies for personalized treatment against MASLD.[45]

The current understanding is that dietary fat, FFAs from adipose tissue, and *de novo* liver lipogenesis triggered by carbohydrates, are the main contributors to the pathophysiology of MASLD. These factors may further contribute to the progression of fibrosis by inducing insulin resistance and inflammation. Our system demonstrates that FFAs alone are sufficient to realistically mimic MASLD *in vitro*. In future studies, our system will be used to investigate the precise role of other substrates, including high levels of glucose, fructose, and cholesterol in the pathogenesis of MASLD. At the current stage, this platform represents a pre-clinical validation system for addressing efficacy and toxicity of MASLD drugs in a human setting, thereby potentially reducing the reliance on animal experiments and helping to guide clinical trials.

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Abbreviations

DEGs, differentially expressed genes; FAO, fatty acid oxidation; FFA, free fatty acid; IR, insulin receptor; LASSO, least absolute shrinkage and selection operator, LD, lipid droplet; MASLD, metabolic dysfunction-associated steatotic liver disease; MASH, metabolic dysfunction-associated steatohepatitis; OA, oleic acid; OCR, oxygen consumption rate; PA, palmitic acid; PHH, primary human hepatocyte; RNA-Seq, RNA sequencing, TG, triglyceride.

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Conflict of interest

The authors declare no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

AZ and YK conceived the study. AZ designed and directed the project, prepared, and wrote the manuscript. YK designed, performed, analyzed, interpreted data, prepared, and wrote the manuscript. PG and AS performed bioinformatics analyses of RNA sequencing and WGCNA. SW performed and analyzed Seahorse Mito-stress assay. JT contributed to bioinformatics analyses using GENVESTIGATOR. KM, RS, WA, and CC helped establish the human hepatocyte culture. JH provided PHH and co-developed the culture system. All authors contributed to data collection and interpretation. YK, PG, JH, AS, and AZ critically revised the manuscript for intellectual content.

Data availability statement

Whole data from RNA-Seq and WGCNA are available in the gene expression omnibus (GEO), accession number GSE247407 and can be found online at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE247407.

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Supplementary data

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