Comparing an *in vitro* Organ-on-Chip model of Non-Alcoholic Steatohepatitis to murine models and liver tissue from patients

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INTRODUCTION

As a result of the increased prevalence of diabetes, obesity and metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) is now the most common chronic liver disease in developed countries (1). NAFLD is a spectrum of pathologies ranging from benign hepatic steatosis through to non-alcoholic steatohepatitis (NASH), which can ultimately lead to cirrhosis and liver cancer. NASH is a serious condition, defined as a combination of hepatic steatosis, inflammation, hepatic damage and pericellular liver fibrosis. There are currently no FDA approved drugs for the treatment of NAFLD/NASH and there is a clear requirement for better models to understand this disease.

Currently available preclinical models, be it *in vivo* or *in vitro*, have a range of limitations and do not fully represent the key aspects of the human disease state. In particular many preclinical rodent models have been developed, but no individual model fully recapitulates the human disease and significant differences exist in the transcriptomic profile of the liver tissue, the manner in which triglycerides accumulate within the liver, and the level of hepatic fibrosis (2). Studying NASH using advanced human *in vitro* models offers the ability to perform studies at the cellular level allowing molecular mechanisms to be elucidated and the genetic drives of the disease to be specifically explored. We have investigated here a fully human *in vitro* NASH model as a potential solution to improve disease understanding. The model is a co-culture of primary human hepatocytes (PHH), Kupffer (HK) and hepatic stellate cells (HSC) cultured in variety of conditions including +/- excess sugar, fat or exogenous TGFβ. These co-cultures are maintained in 3D microtissues in an organ-on-chip (OOC)/ microphysiological system (MPS) which uses microfluidics to perfuse culture medium across the microtissues.

AIM

To explore the transcriptional, soluble biomarker and fibrosis phenotype of a fully human *in vitro* organ-on-chip NASH model and compare it to profiles from NASH patients and murine models. We have also validated the model for use in NASH drug discovery by analysing the effects of Obeticholic acid and Elafibranor on disease endpoints, whilst ensuring that dosing of compounds used are physiologically relevant and translational.

MATERIALS & METHODS

Cryopreserved primary human hepatocytes (PHH), human Kupffer cells (HK) and human hepatic stellate (HSC) cells were obtained from Life Technologies (USA). 0.6 x 10^6 hepatocytes were seeded into each well on the PhysioMimix™ OOC LC-12 plate (CN Bio Innovations Ltd) along with 0.06 x 10^6 Kupffer and 0.06 x 10^6 HSCs (Steatosis conditions contained 10-fold less HK and HSC). Cells were cultured in HEP-Lean or HEP-Fat medium which contains physiologically relevant concentrations of glucose, insulin, and high concentrations of saturated and unsaturated free fatty acids. Cells were cultured in the PhysioMimix™ platform for up to 15 days.

Fat accumulation was measured by Oil Red O staining of fixed microtissues, with images acquired on a Nikon Eclipse Ti-E inverse fluorescent microscope. Staining was quantified by absorbance at 515 nm and normalised to total protein content, measured by BCA assay (ThermoFisher). Production of IL-6, TIMP1, Fibronectin and albumin were all measured by ELISA (R&D systems). Cytokine profiles were also analysed in cell culture samples by Luminex analysis using Milliplex map Human cytokine/chemokine magnetic bead panel – premixed 38 plex (Merck Millipore).

To measure fibrosis in 3D microtissues, scaffolds were stained with antibodies against α-SMA and collagen type 1. Phalloidin was used to stain actin and DAPI stained nuclei. Using a Yokogawa CV7000 high content imaging system, 8 areas were selected per scaffold and high res Z-stack images acquired. Data generated was quantified for each marker using automated MatLab scripts. For transcriptomic analysis RNA-seq was used to analyse cDNA generated from total RNA extracted from scaffolds using TRIzol. cDNA samples were analysed using an Illumina NextSeq500 benchtop sequencer and data was initially collated by KAPA Hyper mRNA Library Prep. Lists of differentially expressed genes were generated for each condition by comparing to control samples and these were compared to published literature and online databases.

ACKNOWLEDGMENTS

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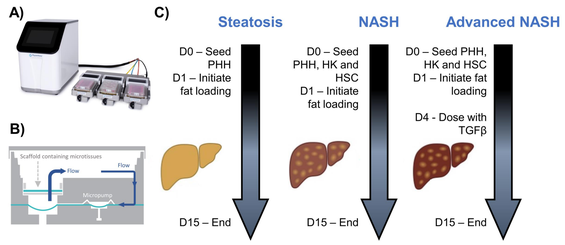

Figure 1 – Human *in vitro* OOC NASH model set up

A) The *in vitro* model utilises the PhysioMimix™ OOC cell culture system, B) which uses open well plates designed for the culture of primary liver cells in 3D in an engineered scaffold. The scaffolds are continually perfused with cell culture medium through the experiment to provide biomechanical stimuli, as well as to provide nutrient and oxygen provision (3). C) The NASH model can be generated using this set up in three ways: 1) culturing PHH alone (mono-culture) in high fat medium a model of steatosis is generated (no inflammation or fibrosis); 2) culturing PHH, HK and HSC together (co-culture) in the model in high fat medium generates a NASH phenotype including fibrosis and inflammation; 3) the phenotype of the model can be further enhanced using exogenous factors such as TGFβ.

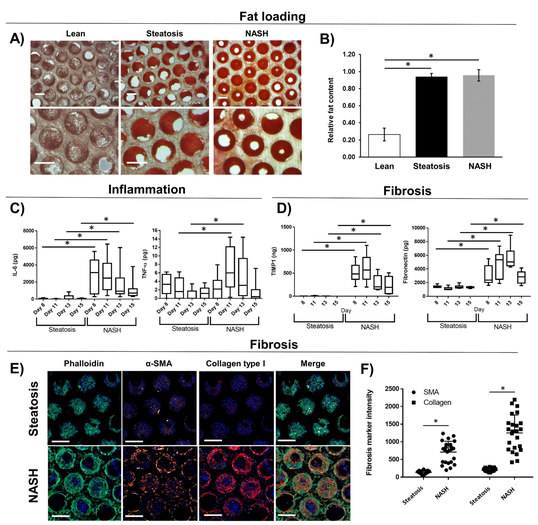

Figure 2 – Co-culture microtissues loaded with fat have inflammatory, steatotic and fibrotic NASH characteristics

PHH, HK and HSC were cultured together for fifteen days in either lean or fat conditions, alongside steatosis controls. A) Cells were analysed/imaged for intracellular fat accumulation. B) Fat loading was measured and quantified by Oil Red O staining. C) Inflammatory biomarkers were measured in cell culture medium; D) Fibrosis biomarkers were also measured in cell culture medium. E) Fibrosis and the presence of extracellular matrix proteins was measured and quantified using high content confocal microscopy. Microtissues were imaged (representative images shown) and F) images quantified. All scale bars 300 µm. Data are mean ± SD, n = minimum 6. * = P < 0.05.

RESULTS

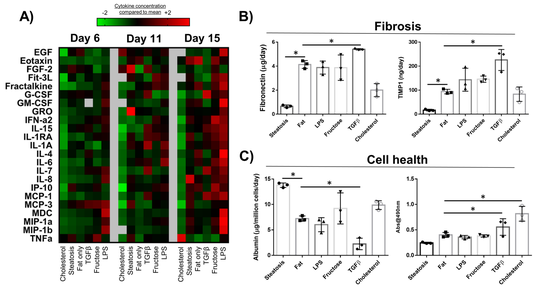

Figure 3 – Soluble biomarkers expressed in OOC NASH model vary dependent on additional cues

PHH, HK and HSC were cultured for 15 days under fat conditions (NASH condition) and compared to steatosis controls. NASH cultures were also cultured with or without LPS, fructose, cholesterol and TGFβ to alter the phenotype of the model. A) Luminex analysis was used to quantify concentrations of human cytokines/chemokines in culture medium samples from the OOC platform. Data shown are a mean for each condition expressed compared to the overall mean expression for each cytokine. B) TIMP1 and Fibronectin fibrosis biomarkers and C) albumin and LDH cell health biomarkers were measured in culture medium by ELISA or colorimetric assay; and are shown as a mean ± SD at Day 15. All data are a minimum of n = 3. * = P < 0.05.

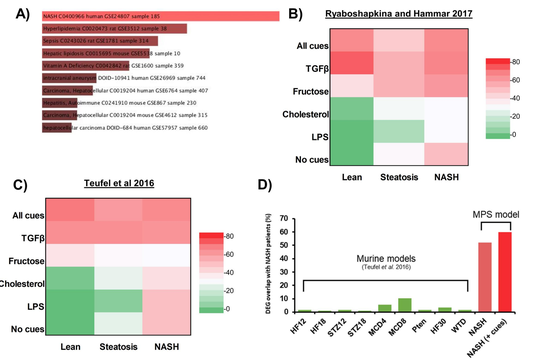

Figure 4 – Transcriptomic profile of NASH model correlates with expression from human samples but not murine models

PHH, HK and HSC were cultured for 15 days under fat conditions (NASH condition) and compared to steatosis controls or lean controls. All cultures were also performed with or without LPS, fructose, cholesterol and TGFβ to alter the phenotype of the model. A) Transcriptomic profile from NASH model (no additional cues) was compared to the Human Gene Atlas database (www.ebi.ac.uk/gxa/home) and identified hits are shown graphically. B) The transcriptional profiles of the model were compared to the 218 gene list identified by Ryaboshapkina and Hammar (4) as being key gene changes in human NAFLD/NASH. Heatmap represents percentage of genes that change expression compared to published list. C) The transcriptional profiles of the model were compared to the 193 gene list identified by Teufel *et al.* (5) as being key gene changes in human NAFLD/NASH. Heatmap represents percentage of genes that change expression compared to published list. D) Teufel et al. (5) compared the expression of the same 193 genes to 9 murine NASH models and the graph shows the number of correctly expressed genes in each murine model and in the OOC NASH model, either with or without additional cues.

	Obeticholic acid	Elafibranor
Non-specific binding to OOC platform across 72 h exposure	<0.1 %	<0.1%
Protein binding in fat cell culture media	>99%	98%
$T_{1/2}$ in OOC platform	12 h	16 h
Clinical dose	10 mg or 25 mg dose QD	80 mg or 120 mg QD
Plasma Cmax (and liver Cmax)	0.6 µM (12.5 µM)	13 µM (not known)
Plasma protein binding	>99%	Not available
Proposed dosing strategy for OOC model	Dosed daily into OOC at 0.6 µM, 5.0 µM and 12.5 µM	Dosed daily into OOC at 1.3 µM, 13 µM and 65 µM

Table 1 – Determining scaled dosing strategy for studies with Obeticholic acid and Elafibranor in OOC in vitro NASH model

Non-specific binding to the OOC platform was determined by incubating compounds in OOC for 72 hours at 1 µM. Protein binding of compounds in fat medium was determined using rapid equilibrium dialysis (RED) analysis. The metabolic rate for each compound was determine using PHH, HK and HSC co-cultures cultured in the OOC platform for 8 days and then exposed to 1 µM compound for 48 hours. For all studies samples were taken across appropriate time courses and measured using quantitative LC-MS/MS. All data are a mean, N=3. Clinical data for OCA was obtained from Edwards *et al.* (6) and for ELF from Staels *et al.* (7)

Figure 5 – Obeticholic acid and Elafibranor reduce NASH phenotype in co-culture microtissues

PHH, HK and HSC were cultured for 15 days under fat + TGFβ conditions (TGFβ added from day 4 onwards). After 4 days cultures were dosed with Obeticholic acid (OCA), Elafibranor (ELF) or vehicle (0.1% DMSO) every day. A) Key cytokines expressed in different conditions at two timepoints during experiment, shown as Log2 fold change compared to vehicle control samples. B) IL-6 production at day 15 as measured by ELISA. C) Confocal microscopy images of microtissues from each condition at the end of study (representative images shown). For each scaffold 8 regions of interest acquired and each biological condition has N=4. D) Quantification of staining for collagen type 1 and alpha-SMA in microtissues. Data are mean ± SD, n = 4. * = P < 0.05.

CONCLUSIONS

Utilising the organ-on-chip PhysioMimix™ platform we have generated a fully human in *vitro* model of NASH. Co-cultures of PHH, HK and HSC were cultured in fat containing medium which induced key features of clinical disease including fat loading, inflammation and fibrosis. In particular, extra-cellular fibrotic proteins were quantified using an automated unbiased confocal microscopy approach. To further explore the phenotype of the model the soluble biomarker profile of the model was assessed when the model was exposed to range of different biological cues. The use of LPS, fructose and TGFβ were all observed to drive an enhanced inflammatory profile in the model, but TGFβ also enhanced the fibrotic phenotype by enhancing TIMP1 and fibronectin expression. We then compared the transcriptional profile of the model following the addition of various biological cues and compared the expression of key genes associated with human NAFLD/NASH development as previously identified (4-5). Under particular conditions the model was shown to have a high correlation of expression of the disease associated genes (>65%), this could either be induced through a dietary model (just fat) or through the use of TGFβ. When explored against the human gene atlas the fat only NASH model was also found to correlate with an expression profile that matches human NASH. This data demonstrates that the OOC model presents a highly similar phenotype and transcriptional profile to human NASH and this phenotype can be modulated through the use of different biological cues, in particular TGFβ which will induce an enhanced fibrotic phenotype. We determined a scaled dosing strategy for OCA and ELF in the OOC model to closely mimic clinical dosing of these compounds. Both were dosed into the OOC NASH model and demonstrated efficacy similar to what is observed *in vivo*. Both molecules were observed to reduce a range of inflammatory cytokines and additionally reduced the expression of key fibrotic markers in a dose dependent manner. This work demonstrates that the OOC *in vitro* NASH model is ideally suited to exploring the molecular mechanisms that underlie the development of human NASH and is a useful tool for analysing the efficacy of novel anti-NASH therapeutics against a range of different target pathways.

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