Fully human *in vitro* gut-liver model of non-alcoholic steatohepatitis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a wide spectrum of liver abnormalities with the most severe cases involving progressive liver injury in the form of non-alcoholic steatohepatitis (NASH). Key characteristics of NASH include lipid accumulation in hepatocytes, chronic inflammation and extra-cellular matrix deposition, leading to fibrosis (1). NASH is the liver manifestation of the metabolic syndrome, with close association with insulin resistance, obesity and Type 2 diabetes. The interaction between the liver and the gut (the "gut-liver axis"), is now known to play a critical role in NAFLD onset and progression. Dietary factors and gut microbiota can both alter intestinal barrier function, leading to metabolic endotoxemia and low-grade inflammation which will contribute to the development of NAFLD (2). There is a requirement for better pre-clinical models to understand the molecular mechanisms underlying NAFLD/NASH (3). Particularly models that mimic disease progression in the liver with linked interactions to the gut, adipose tissue and immune system will have the highest translational relevance. This is particularly prevalent as there remains no FDA approved drugs for the treatment of NAFLD/NASH.

AIM

Using perfused microphysiological cell culture systems, we aimed to develop a fully human *in vitro* NAFLD/NASH model, utilising primary human hepatocytes (PHH), Kupffer (HK) and hepatic stellate cells (HSC). The complexity of this model was further enhanced by combining the NASH liver model with an *in vitro* gut model to allow the interactions of the two organs to be studied.

MATERIAL & METHODS

Cryopreserved human hepatocytes and Kupffer cells (Life Technologies) and hepatic stellate cells (isolated by Krista Rombouts - UCL) were seeded together into LiverChip® wells. Cells were cultured in chemically defined lean or fat media containing standard supplements, including physiological concentrations of glucose, insulin, ± 600 µM free fatty acids. Media was changed on wells every 2-3 days.

Production of IL-6, TNFα, pro-collagen (R&D systems) and albumin (Assay Pro) were all measured by ELISA. Kupffer and stellate cells were transduced with Adenoviruses expressing eGFP and mCherry (VectorLabs) (MOI: 25) prior to seeding into LiverChip®. Microtissues were imaged on a Nikon Eclipse Ti-E inverse fluorescent microscope. Total RNA was extracted using TRIzol (Ambion) and RNA was converted to cDNA using the high capacity cDNA synthesis kit (Life Technologies). Gene expression profiles were quantified in equivalent cDNA samples using Taqman assays on an ABI QuantStudio 6 real time PCR system.

Caco-2 cells were pre-cultured on 0.33 cm^2 transwells for 21 days before being combined with liver cultures in T12 platforms. TEER measurements of Caco-2 barrier integrity were made using a EVOM2 epithelial voltohmmeter (World Precision Instruments). LDH release was measured using the Cytox96 assay kit (Promega). Caco-2 transwells were dosed apically with 2.5 mM 7-hydroxy-coumarin (7-HC) and the concentration in the basolateral side was measure by a fluorometer (BioTek). Caco-2 cells were dosed basolaterally with recombinant 2.5 ng/ml TNFα and 10 ng/ml IFNγ (R&D systems)

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RESULTS

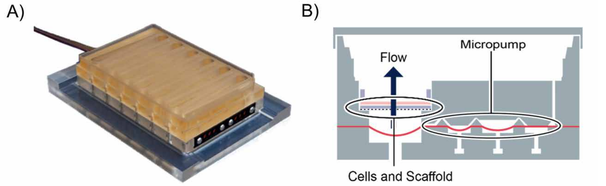

Figure 1 – LiverChip® Hardware
A) LiverChip® is a 12 well perfused cell culture system. B) Media flows through the engineered scaffold containing microtissues due to the action of a pneumatically operated micropumps. The speed and direction of flow can be adjusted using an electronic controller.

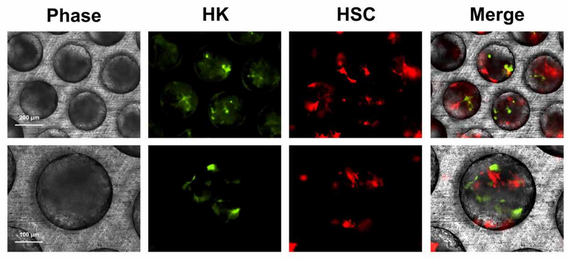

Figure 2 – Triple-culture of Hepatocytes, Kupffer and Hepatic Stellate cells
PHH, Kupffer cells (HK) and hepatic stellate cells (HSC) were all seeded into the LiverChip® platform and cultured together for 4 days. Prior to seeding HK and HSC were transduced with adenoviruses expressing eGFP and mCherry respectively. Scaffolds containing microtissues were imaged by fluorescent microscopy to demonstrate localisation of HK and HSC, representative images are shown at two magnifications.

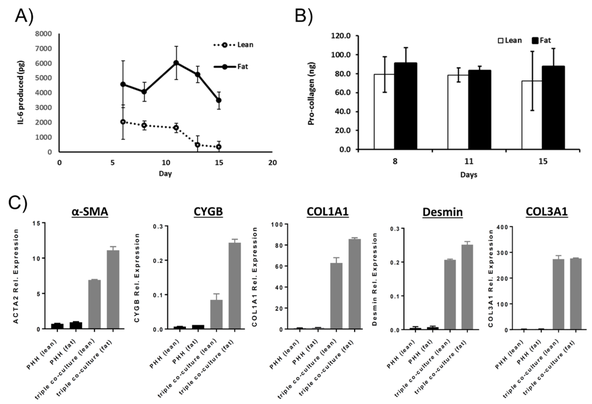

Figure 3 – Triple-culture loaded with fat induces inflammatory and fibrotic NASH characteristics
PHH, HK and HSC were cultured together in LiverChip platform for fourteen days and in either lean or fat conditions. A) IL-6 production and B) pro-collagen-1 were measured in the culture medium. C) Expression of fibrotic marker genes was analysed, normalised to GAPDH housekeeping gene and compared to monocultures of PHH cultured in the same media conditions. Data are mean ± SD, n = 3.

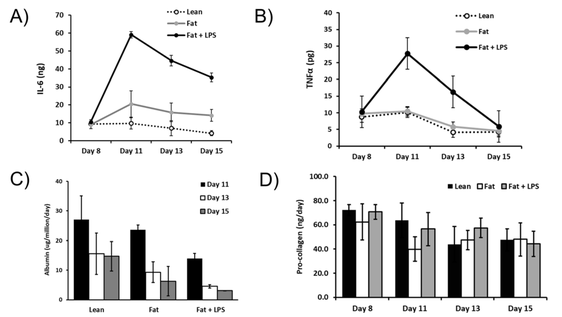

Figure 4 – Stimulation with Lipopolysaccharide further enhances NASH phenotype of triple-culture
PHH, HK and HSC were cultured for 14 days under fat and lean conditions in the LiverChip® platform. After 8 days some fat cultures were additionally dosed with 0.5 ng/ml LPS every 2 - 3 days. A) IL-6 production, B) TNFα production, C) Albumin production and D) pro-collagen-1, were all measured in the cell culture medium. Data are mean ± SD, n = 3.

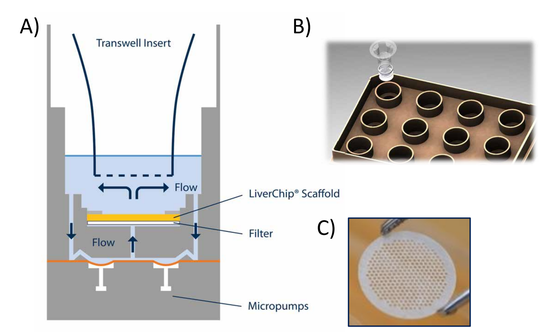

Figure 5 –T12 multi-organ MPS hardware.
The T12 plate is a 12 well perfused cell culture system, that allows that culture of three dimensional PHH cultures alongside a second organ. The system has a reduce working volume <400 µl to allow for scaled interactions between organs. A) Media is recirculated in each well by two micropumps which move the media through the PHH containing scaffold. B) Transwells sit in the top of each well and can be used to culture a variety of cell types. C) LiverChip® scaffold use to culture 3D PHH in the T12 plate, each scaffold cultures 600k PHH.

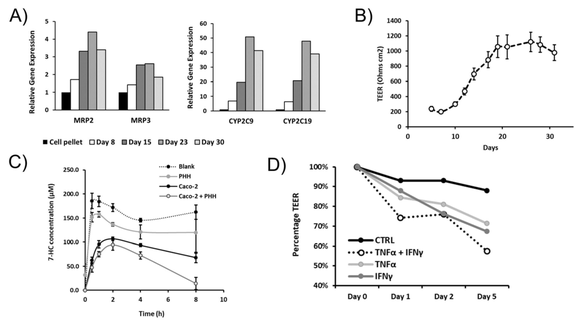

Figure 6 – Functional Caco-2 gut model can be damaged by inflammatory stimuli
Caco-2 cells were matured on transwells for 30 days, A) expression of key genes was analysed during maturation. Data was normalised to GAPDH housekeeping gene and compared to freshly thawed cells. B) TEER measurement for epithelial integrity. C) Matured Caco-2 cells were combined with PHH cultures in an MPS platform and dosed apically with 7-HC. 7-HC absorption and metabolism was determined by fluorescence. D) Matured Caco-2 cells were dosed with recombinant inflammatory cytokines and TEER measured after dosing. Data are mean ± SD, n = 3.

RESULTS

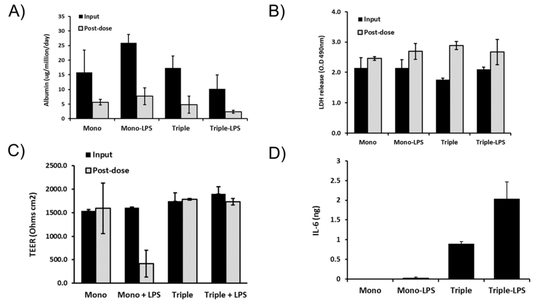

Figure 7 – Gut-liver NASH model with inflammatory crosstalk
PHH, HK and HSC (Triple) or PHH (Mono) were cultured for five days in fat media in the LiverChip® platform. Caco-2 cells were matured for 21 days on transwells. Cultures were combined in the T12 platform and cultured for a further 3 days. Caco-2 cells were additional dosed apically with 100 ng/ml LPS to mimic endotoxin release from the gut. Cytotoxicity in the system was determined by A) measuring Albumin release from hepatocytes, and B) measuring LDH release in the system before (input) and after LPS dosing. C) The effect on gut epithelia integrity was measured by analysing TEER. D) Inflammation in the system was determined by measuring IL-6 in the basolateral cell culture medium after LPS dosing. Data are mean ± SD, n = 3.

CONCLUSION

Utilising perfused MPS culture platforms we have generated fully human models of NASH liver and NASH gut/liver. Co-cultures of PHH, HSC, HK were cultured in the presence of free fatty acids for 14 days in a 3D perfused MPS platform and the phenotype of the model represent in vivo human NASH. Cells in the system accumulate intracellular fat, produce inflammatory markers and upregulate markers of fibrosis. The phenotype of co-culture can be further enhanced by dosing of low concentration LPS to mimic endotoxin release.

We additional established a Caco-2 gut model and an MPS platform to allow the gut and liver cultures to interact. The gut cultures were shown to be susceptible to inflammatory stimuli that reduces gut integrity, a process know to occur in patients with NASH. Combining the gut and NASH liver models we demonstrate gut-liver crosstalk where by inflammation from the NASH liver was increased when LPS leaked from gut epithelium.

These *in vitro* NASH models are ideally suited to exploring the molecular mechanisms that underlie the development of human NASH and will be a useful tool for analysing the efficacy of novel anti-NASH therapeutics against a range of different target pathways.

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