The PNPLA3 I148 variant enhances the disease phenotype of hepatic stellate cells in an *in vitro* model of non-alcoholic fatty liver disease

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is now considered the most predominant cause of chronic liver disease in the Western world and its worldwide prevalence continues to increase (1). NAFLD includes 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, collagen deposition and 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.

The single nucleotide polymorphism I148M in the gene encoding patatin-like phospholipase domain–containing protein 3 (PNPLA3) is strongly associated with adverse outcomes for NAFLD patients. PNPLA3 I148M is associated with increased hepatic inflammation, steatosis and enhanced development and severity of liver fibrosis (2). The mutation is proposed to directly affect primary human hepatocyte (PHH) propensity to become steatotic and the fibrogenic phenotype of hepatic stellate cells (HSC) (2). Primary I148M HSC have been shown to demonstrably produce more inflammatory mediators, and have higher lipid droplet content than equivalent wild-type (WT) HSC (3).

PNPLA3 I148M effects in HSC have only been explored in isolation and not in a NAFLD disease model. Using a fully human in vitro NAFLD triple co-culture model, which comprises PHH, Kupffer (HK) and HSC, we investigated how PNPLA3 I148M in HSC influenced NAFLD development.

Aim

Using the organ-on-a-chip technology PhysioMimix™, we have developed a fully human *in vitro* NAFLD/NASH model, utilising multiple primary human liver cell types. To demonstrate the utility of this model, we have analysed how the I148M mutation in the PNPLA3 gene carried specifically in HSC affects the disease morphology of the model. Mutations in this gene are associated with disease progression and have been recently shown to directly influence HSC disease phenotype.

Methods and materials

Cryopreserved human hepatocytes and human Kupffer cells were obtained from Life Technologies (USA). Hepatic stellate cells were isolated and cryopreserved by Krista Rombouts (UCL). 0.6 x 10^6 hepatocytes were seeded into each well on the PhysioMimix™ LC-12 plate, alongside 0.06 x 10^6 Kupffer and 0.06 x 10^6 HSC. Cells were cultured in lean or fat DMEM containing standard supplements, including physiologically relevant concentrations of glucose, insulin, and ± 600 µM free fatty acids.

Fat accumulation was measured by Oil Red O staining of fixed microtissues. Staining was quantified by absorbance at 515 nm and normalised to total protein content, measured by BCA assay (Thermo Fisher). Production of IL-6, FGF-19 and albumin were all measured by ELISA (R&D Systems). Free fatty acid consumption was measured using a colourmetric assay (Abcam). Cytokine profiles were also analysed in cell culture samples using Bio-Plex Pro™ Human Cytokine 28-plex Assay (Bio-Rad).

To visualise Kupffer and stellate cells in the platform, these cells were transduced with Adenoviruses expressing eGFP and mCherry (Vector Laboratories) (MOI: 25) prior to seeding into PhysioMimix LC-12 plates. 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 or Fatty Liver RT^2 PCR profiler arrays (Qiagen) on an ABI QuantStudio 6 real time PCR system. Mutations in PNPLA3 (I148M variant) were detected using Taqman assay rs738409 on whole genomic DNA extracted using Qiagen DNeasy blood and tissue kit.

Results

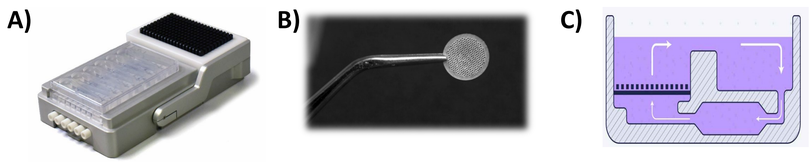

Figure 1 – PhysioMimix™ Hardware
A) PhysioMimix is a perfused cell culture system with open well plates, designed for the culture of primary liver cells in 3D. B) Liver MPS plates contain 12 independent culture wells, each containing an engineered scaffold which cells are seeded onto to form microtissues. C) Schematic cross section of an individual well. Pneumatically operated micropumps embedded within the plate control the flow of media within each well. The speed and direction of flow can be adjusted using an electronic controller.

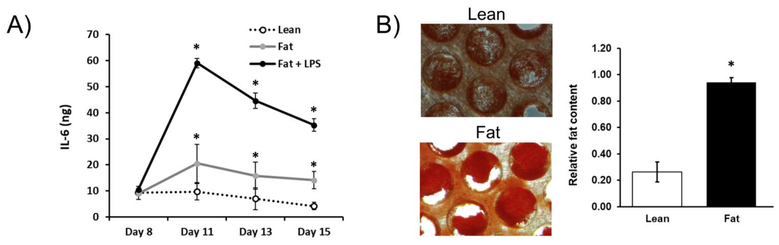

Figure 2 – Co-culture microtissues loaded with fat have inflammatory, steatotic and fibrotic NAFLD characteristics
PHH, HK and HSC were seeded into the platform and cultured together for 15 days in either lean, fat or fat + LPS (0.5 ng/ml) conditions. As a control, PHH monocultures were cultured in parallel. A) Analysis of IL-6 production in the culture medium. B) Fat loading as measured by Oil Red O staining. C) Expression of fibrotic marker genes (in fat loaded cells), normalised to GAPDH housekeeping gene and compared to monocultures of PHH cultured in the same media conditions. Data are mean ± SD, n = 3. * = P < 0.05.

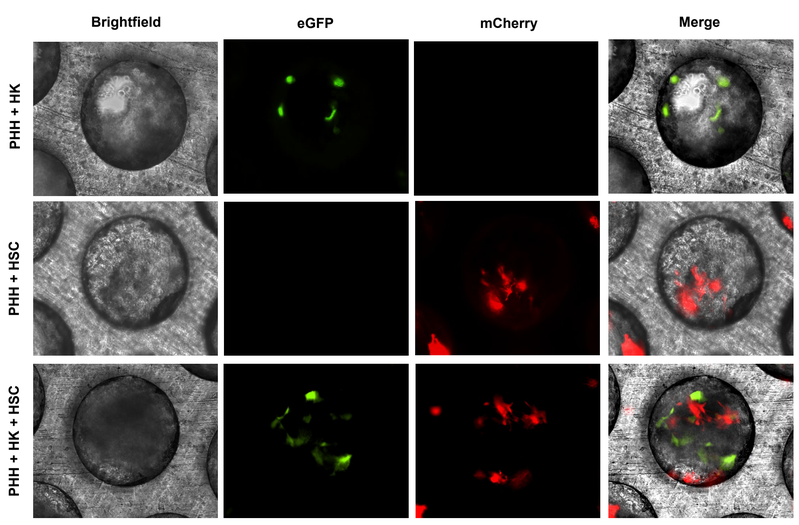

Figure 3 – Co-culture microtissues of hepatocytes, Kupffer and hepatic stellate cells
PHH, HK and HSC were cultured together for 4 days. Prior to seeding, HK and HSC were transduced with adenoviruses expressing eGFP and mCherry, respectively. Scaffolds containing microtissues were imaged by fluorescent microscopy to demonstrate localisation of HK and HSC.

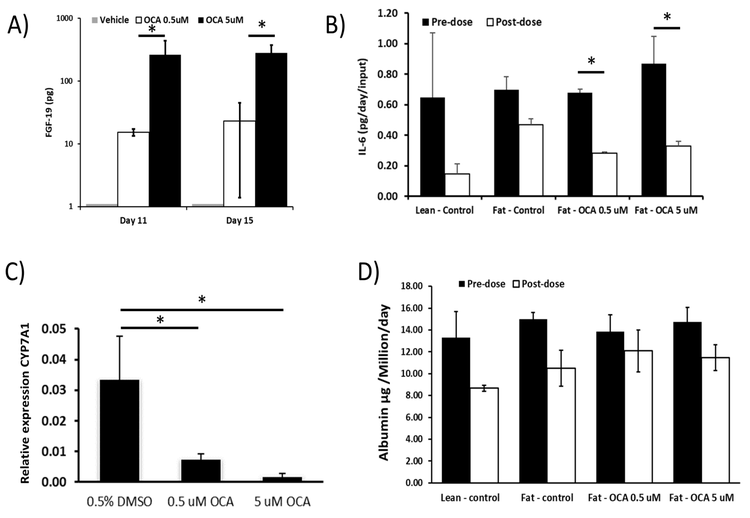

Figure 4 – Obeticholic acid reduces NAFLD phenotype in co-culture microtissues
PHH, HK and HSC were cultured for 15 days under fat and lean conditions. After 8 days some fat cultures were additionally dosed with 0.5 µM or 5 µM Obeticholic acid (OCA) every 2 days. A) FGF-19 production B) IL-6 production, C) Gene expression of CYP7A1 and D) albumin production. Data are mean ± SD, n = 3. * = P < 0.05.

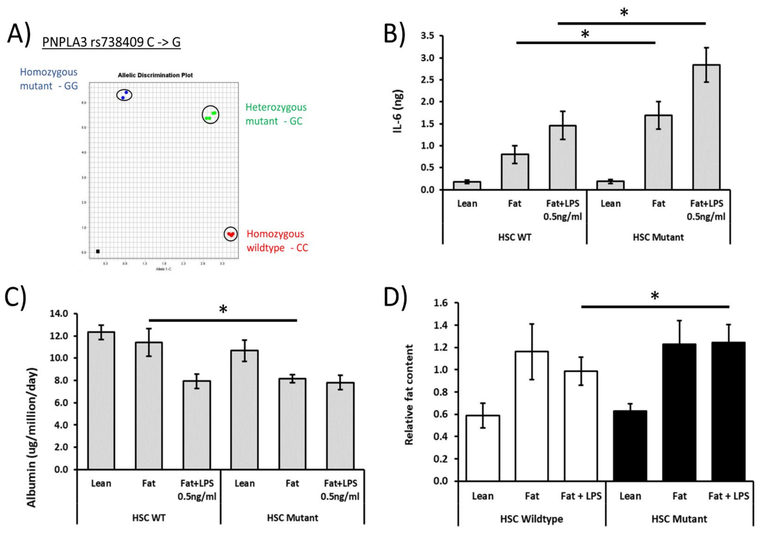

Figure 5 – PNPLA3 I148M variant in hepatic stellate cells promotes the NAFLD phenotype in the co-culture microtissues
PHH, HK and HSC were cultured for 14 days under lean, fat or fat + 0.5 ng/ml LPS conditions. Six different HSC donors were used, three with WT PNPLA3 and three containing the I148M mutation. The same PHH and HK donor cells were used throughout. A) The PNPLA3 I148M mutation in the HSC cells was detected using a Taqman assay. Co-culture phenotype was compared for the different conditions using; B) IL-6 production, C) albumin production, D) fat loading, as measured by Oil Red O staining. Data are taken at day 14 of the culture and are mean ± SD, n = 9. * = P < 0.05.

Fold change in gene expression compared to lean samples:

	HSC WT		*HSC Mutant*	
Gene	**Fat**	**Fat + LPS**	**Fat**	**Fat + LPS**
ACTA2	1.56	1.60	3.517	2.831
CYP2E1	2.31	2.59	2.78	2.56
CYP7A1	-3.68	-6.06	-8.72	-14.97
FASN	-4.93	-7.1	-4.07	-4.35
GCK	-5.17	-10.17	-5.28	-7.06
GK	1.9	2.01	3.43	3.15
IFNG	-3.35	-2.81	13.87	4.04
IGFBP1	1.51	1.32	2.15	2.78
IL6	1.72	1.51	7.88	3.08
IL10	-2.2	-1.26	-2.23	-4.49
LPL	-2.88	-3.38	-5.97	-7.11
PDK4	2.26	2.56	1.27	1.61
SCD	-3.64	-5.41	-3.76	-3.41

Table 1 – PNPLA3 I148M variant in hepatic stellate cells alters gene expression profile of the NAFLD *in vitro* model
PHH, HK and HSC were cultured for 14 days under lean, fat or fat + LPS (0.5 ng/ml) conditions. Six different HSC donors were used, three with WT PNPLA3 and three containing the I148M mutation. The same PHH and HK donor cells were used throughout. Gene expression profiles were analysed in all samples by RT^2 profiler arrays. The expression of each gene was analysed in the Fat and Fat + LPS conditions, and compared to the matched lean condition. Data are taken at day 14 of the culture and are mean ± SD, n = 9.

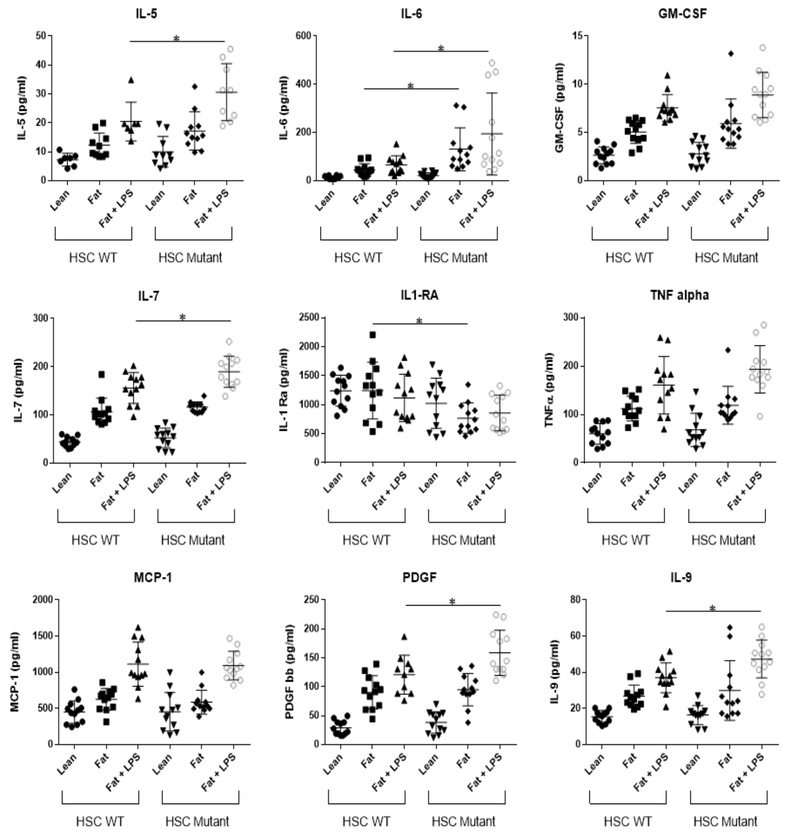

Figure 6 – The cytokine profile in the 3D *in vitro* NAFLD model is altered by fat loading, LPS dosing and the PNPLA3 I148M variant in hepatic stellate cells
PHH, HK and HSC were cultured for 14 days under lean, fat or fat + 0.5 ng/ml LPS conditions. Six different HSC donors were used, three with WT PNPLA3 and three containing the I148M mutation. The same PHH and HK donor cells were used throughout. Cytokine profiles were analysed by multiplex Luminex analysis. Data are taken at day 14 of the culture and are mean ± SD, n = 9. * = P < 0.05.

Conclusion

Utilising the 3D perfused culture platform PhysioMimix™, we have generated fully human model of NAFLD. Co-cultures of PHH, HK and HSC were used to create an immunocompetent *in vitro* liver model that can recapitulate key characteristics of human NAFLD. The co-culture model includes key features of the clinical disease including fat loading, inflammation and fibrosis. The model can be used to probe the effects of anti-NAFLD compounds, as is demonstrated by the use of developmental clinical compound OCA. OCA treatment reduced CYP7A1 expression in the model (key enzyme for cholesterol synthesis) and reduced inflammatory markers.

Utilizing this NAFLD model the effects of the PNPLA3 I148M mutation were investigated in HSC. Different HSC donors were identified that contained either WT or mutant copies of the PNPLA3 gene. When the triple-culture model was run with mutant HSC donors in comparison to the same conditions with WT cells, observations included higher cytokine profiles (particularly after fat and LPS dosing), increased gene expression profiles of disease associated genes and increased levels of fat loading. These findings confirm the direct effect of the PNPLA3 I148M mutation on the disease phenotype generated by HSC.

This work further demonstrates that the 3D *in vitro* NAFLD model is ideally suited to exploring the molecular mechanisms that underlie the development of human NAFLD and is a useful tool for analysing the efficacy of novel anti-NAFLD therapeutics against a range of different target pathways.

References

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