CN-BIO INNOVATIONS

Three-dimensional human cell culture model for studying Non-Alcoholic Fatty Liver Disease

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BACKGROUND

Non-Alcoholic Fatty Liver Disease (NAFLD) is a growing concern worldwide and is set to become the most predominant cause of chronic liver disease. NAFLD describes a range of disease states from liver steatosis, which is the simplest and most common through to late stage liver disease, cirrhosis and liver cancer. There are currently no FDA approved drugs for the treatment of NAFLD and there is a clear requirement for better models to understand disease progression and the molecular pathways underlying the disease (1). Currently available pre-clinical models, be it *in vivo* or *in vitro*, have a number of limitations and differ in regards to the degree of hepatocellular damage and metabolic alterations associated with disease development (2). Current *in vitro* models generally utilise hepatocellular carcinoma-derived cell lines, which lack many of the biochemical properties of primary hepatocytes and fail to represent the complexity of the liver microenvironment and disease pathology (3).

OBJECTIVES

Utilise the perfused cell culture system LiverChip®, to develop a fully human *in vitro* NAFLD model. Culture primary human hepatocytes (PHH) in three dimensions to mimic the liver microarchitecture and support hepatic functions over extended periods of culture, allowing the continual accumulation of fat deposits in the hepatocytes.

MATERIALS & METHODS

Cryopreserved human hepatocytes were obtained from Life Technologies (USA). 0.6 x 10^6 hepatocytes were seeded into each LiverChip® well (3D) in Williams' E medium containing supplements. After 24h of culture cells were changed into fat or lean media, which contained physiologically relevant concentrations of glucose, insulin and fat. Cells were cultured in fat or lean media for up to 14 days with regular media changes. Pioglitazone and Metformin were dosed for 7 days after 7 days pre-culture in fat/lean conditions.

Fat accumulation was measured by Oil Red O staining of fixed micro-tissues. Oil O red stain was removed from tissues and quantified by absorbance at 515 nm. Total protein content of scaffolds was measured by BCA assay (Thermo). Free fatty acid concentration in media was measured by ELISA (AbCam). Adipokine proteome profiler arrays (R&D systems) were used to compare conditioned media samples.

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 Fatty Liver RT2 PCR profiler arrays (Qiagen) on an ABI QuantStudio 6 real time PCR system. P450-Glo (Promega) was used to monitor CYP3A according to the manufacturers' protocol. Simultaneous quantification of seven probe substrate-derived metabolites (cocktail assay) was performed by LC-MS/MS (Xenogesis, Nottingham, UK). 10 uM Diclofenac was dosed for 48h onto PHH and media samples were taken at intervals for quantification by LC-MS/MS analysis (Xenogesis, Nottingham , UK).

RESULTS

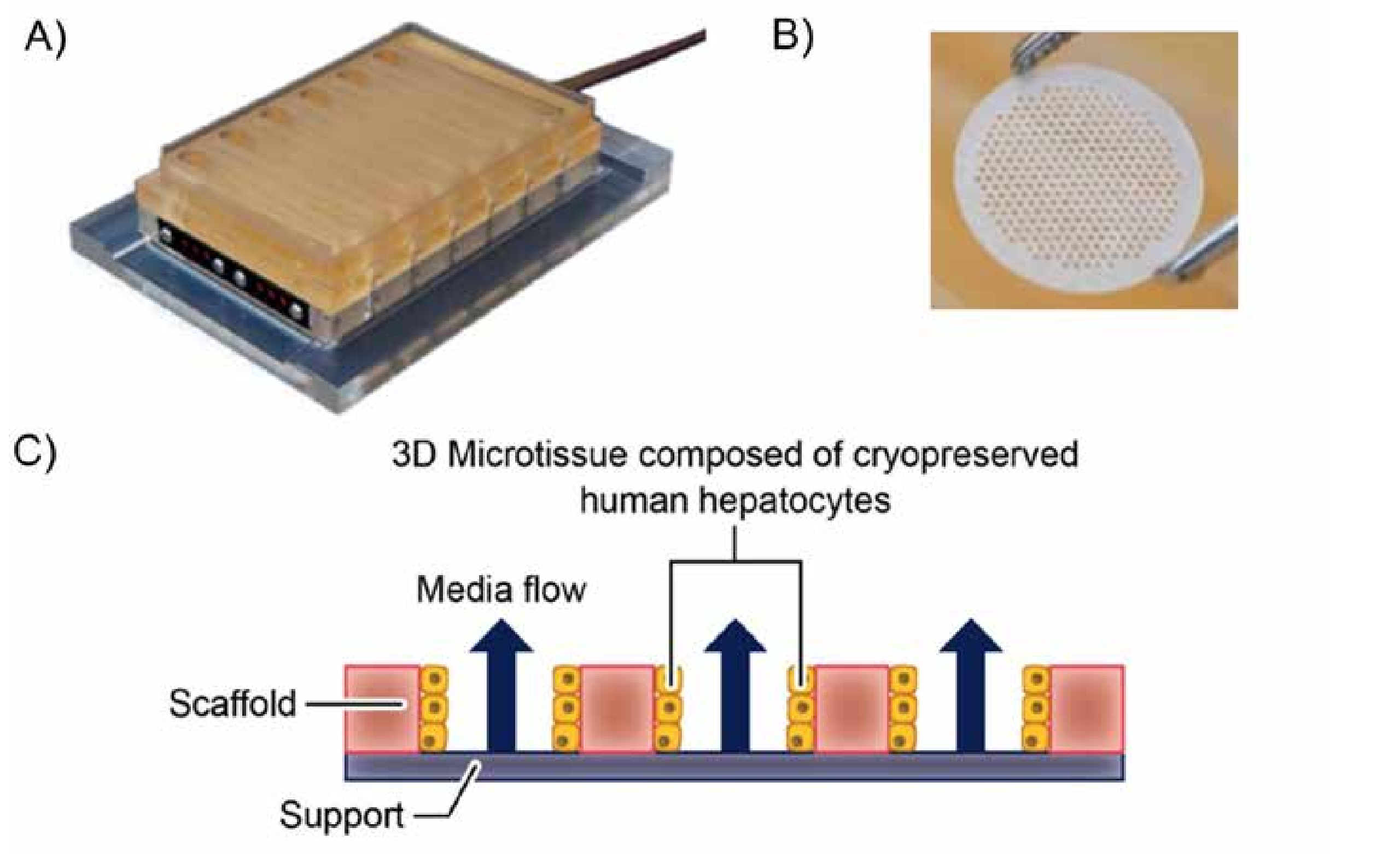

Figure 1: LiverChip® Hardware
A) LiverChip® is a 12 well perfused cell culture system.
B) Hepatocytes form 3D tissue structures in an array of channels through a collagen I-coated scaffold.
C) Media flows through the channels due to the action of a pneumatically operated pumping mechanism. The speed and direction of flow can be adjusted using an electronic controller.

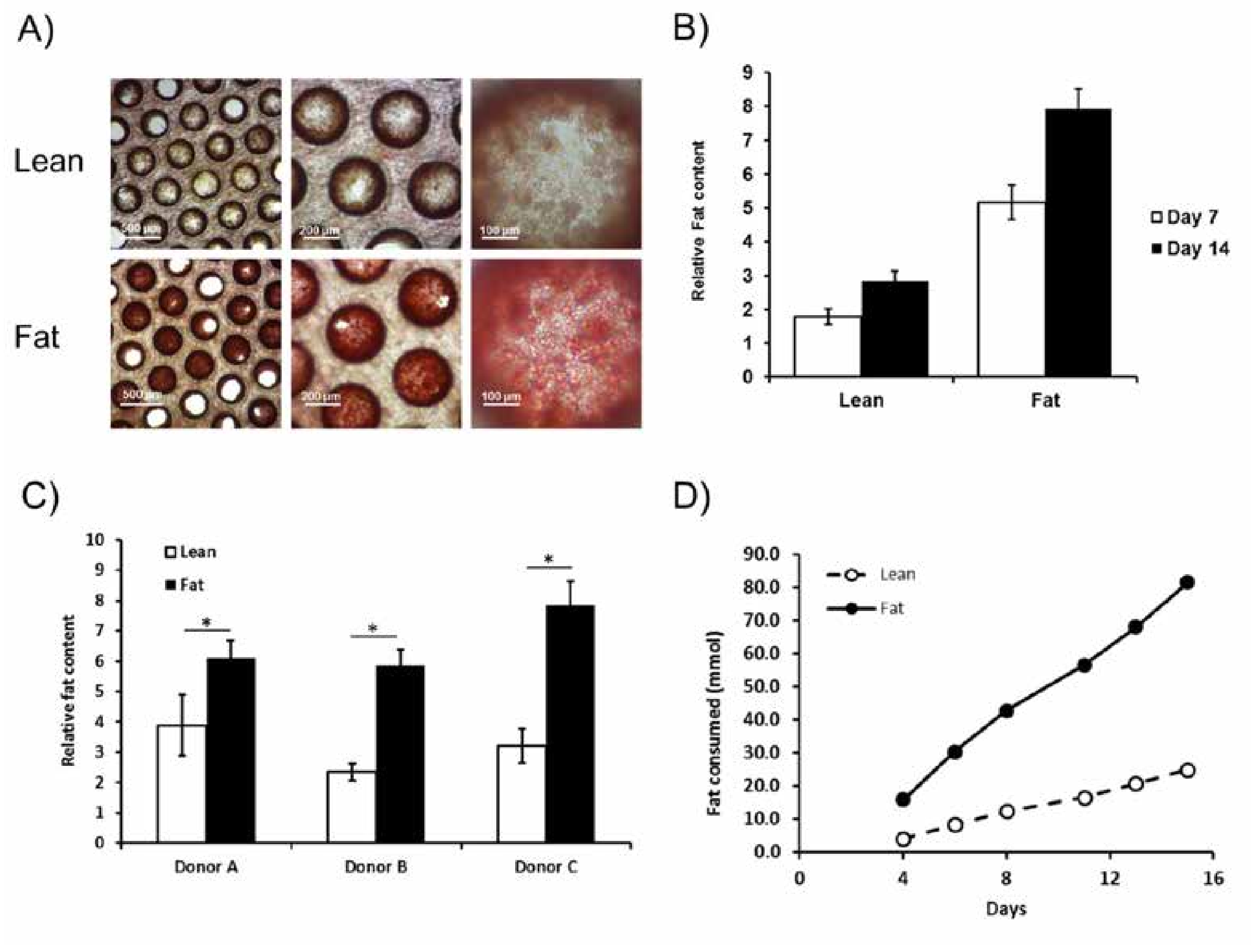

Figure 2 – Hepatocytes accumulate fat over time
PHH were cultured in 3D for 14 days under fat and lean conditions. Fat loading was measured by Oil Red O staining of scaffolds, which was A) observed by microscopy, and B) quantified by absorbance at 515 nm and normalised to total protein content. C) PHH from multiple donors were cultured for 7 days and fat loading measured by Oil Red O staining. D) Fat consumed by PHH over 14 days of culture was calculated by analysing culture medium by ELISA for the presence of free fatty acids. Data are shown as a mean ± SEM; * = P < 0.05.

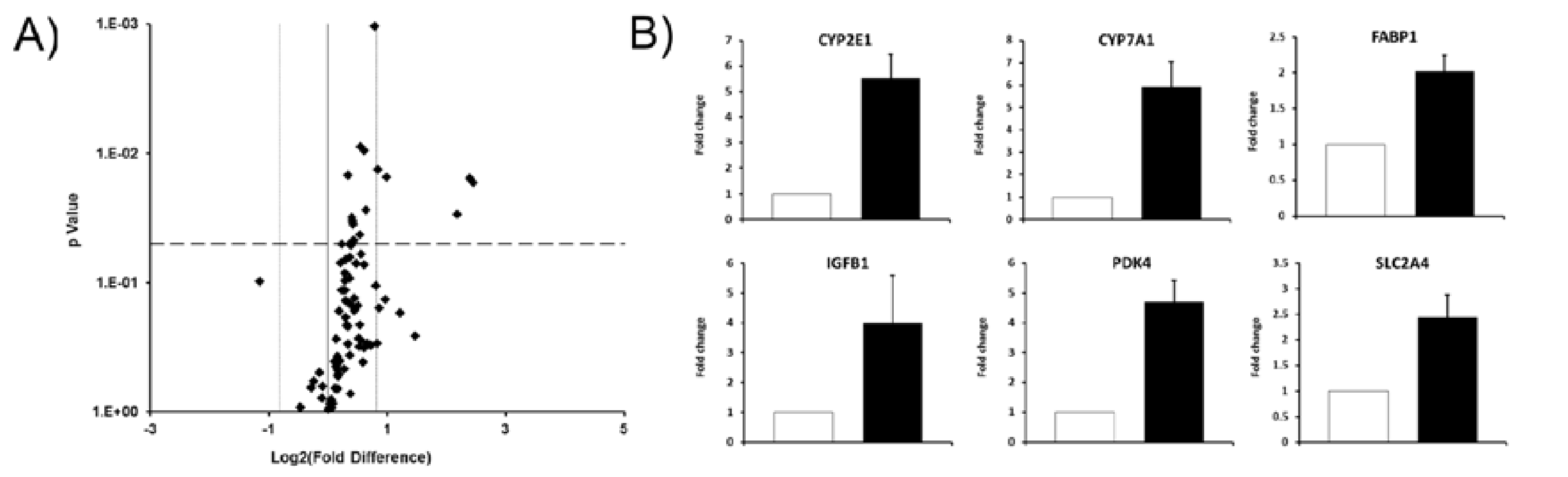

Figure 3 – Hepatocytes cultured in fat media have altered gene expression
PHH were cultured in fat or lean conditions for 7 days before total RNA was extracted and gene expression was compared using Fatty Liver RT^2 Profiler PCR Arrays. A) Gene expression changes were defined by a fold change >1.8 and P = <0.05. Results are an average of three hepatocyte donors (± SEM). B) Fold change in expression in fat vs lean condition of key genes, filled bars = fat, white bars = lean. Data are means ± SEM; P <0.05.

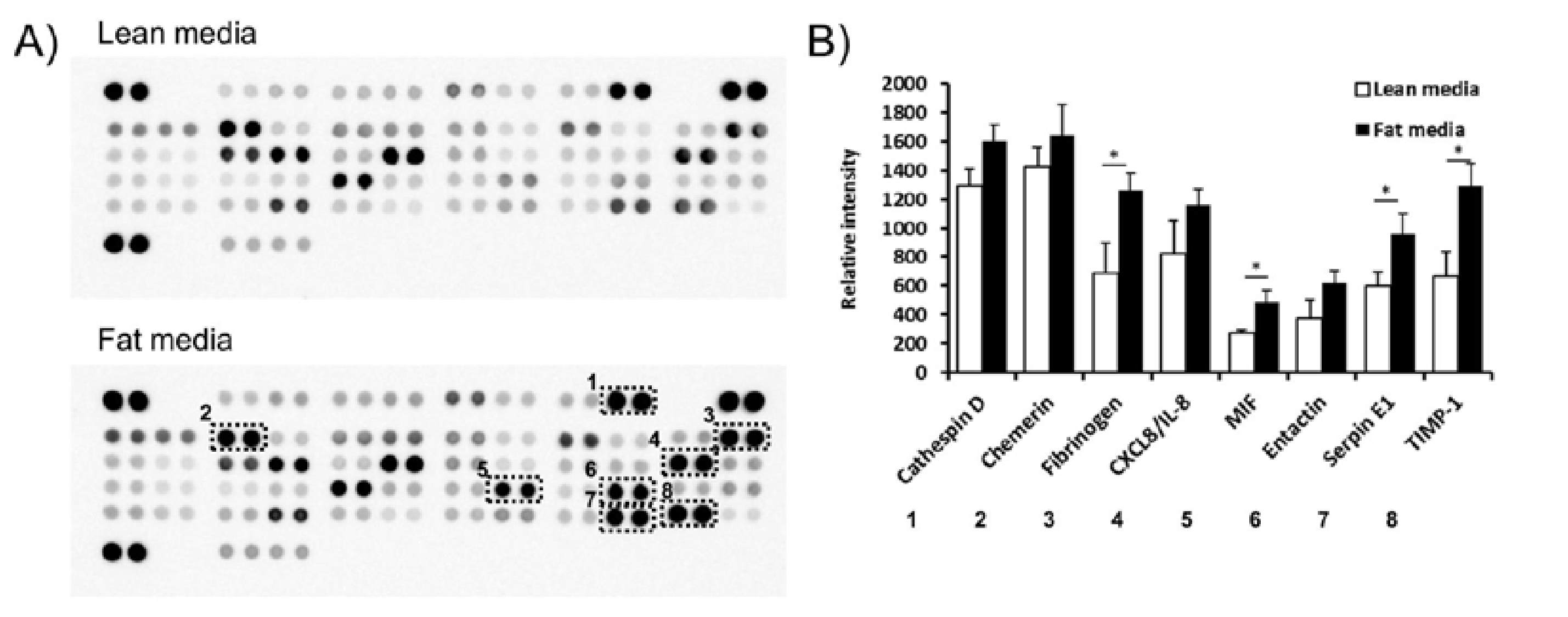

Figure 4 – Hepatocytes cultured in fat media secrete adipokines
PHH were cultured in fat or lean conditions of 7 days and secreted protein production was compared by antibody-based adipokine array analysis (three donors per condition and n=3 per donor). B) Adipokine levels were expressed as the relative densitometric intensity. Data are means ± SEM; P <0.05.

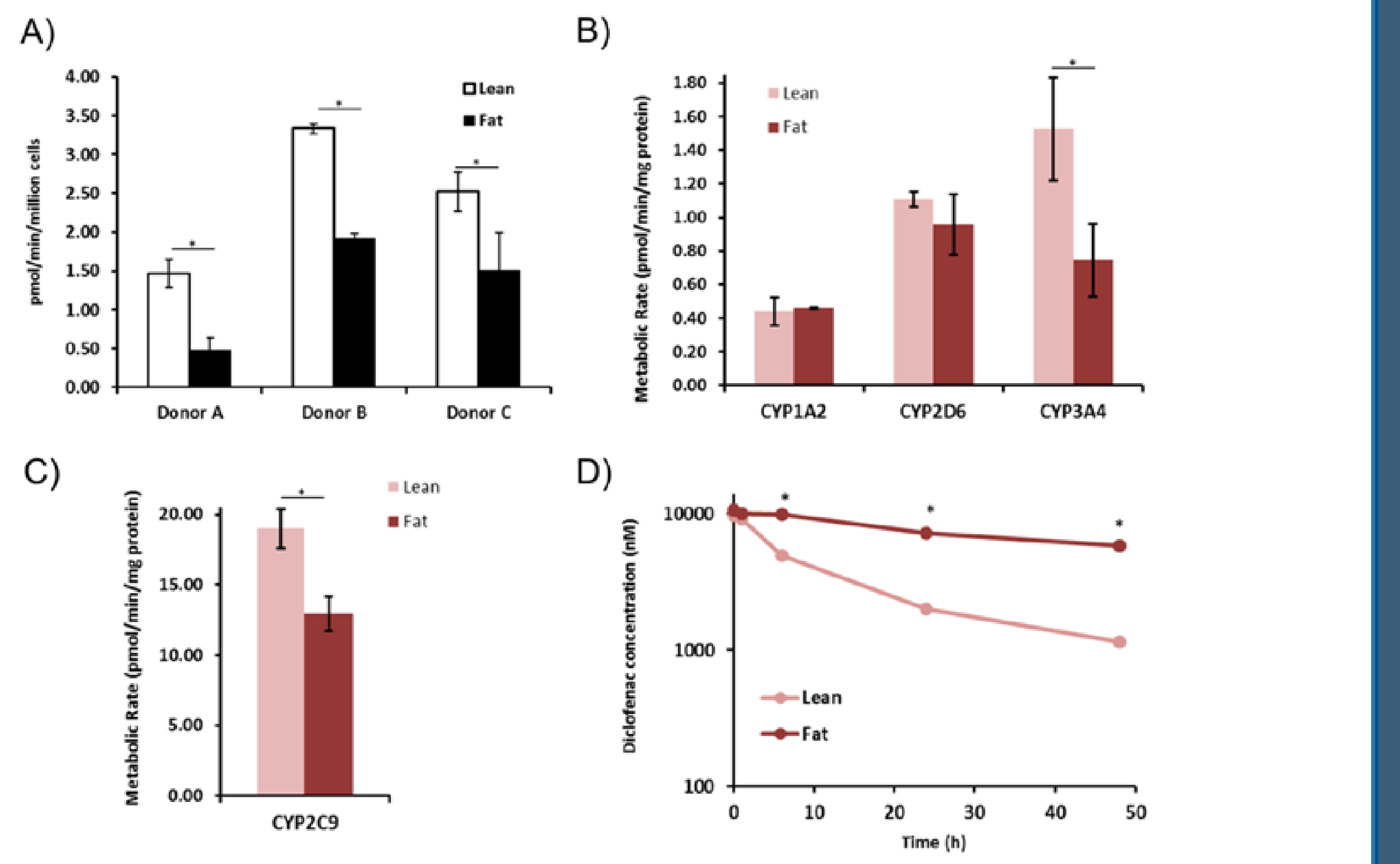

Figure 5 – Fat alters the metabolic capacity of hepatocytes
PHH were cultured in fat or lean conditions for 7 days A) CYP3A activity was measured in three donors by CYP3A-Glo assay. B, C) P450 activity of cells was compared using an LC-MS/MS cocktail assay. D) PHH were dosed with 10 µM diclofenac and samples of culture media analysed by LC-MS/MS. Data is a mean (n=3) ± SEM, P < 0.05.

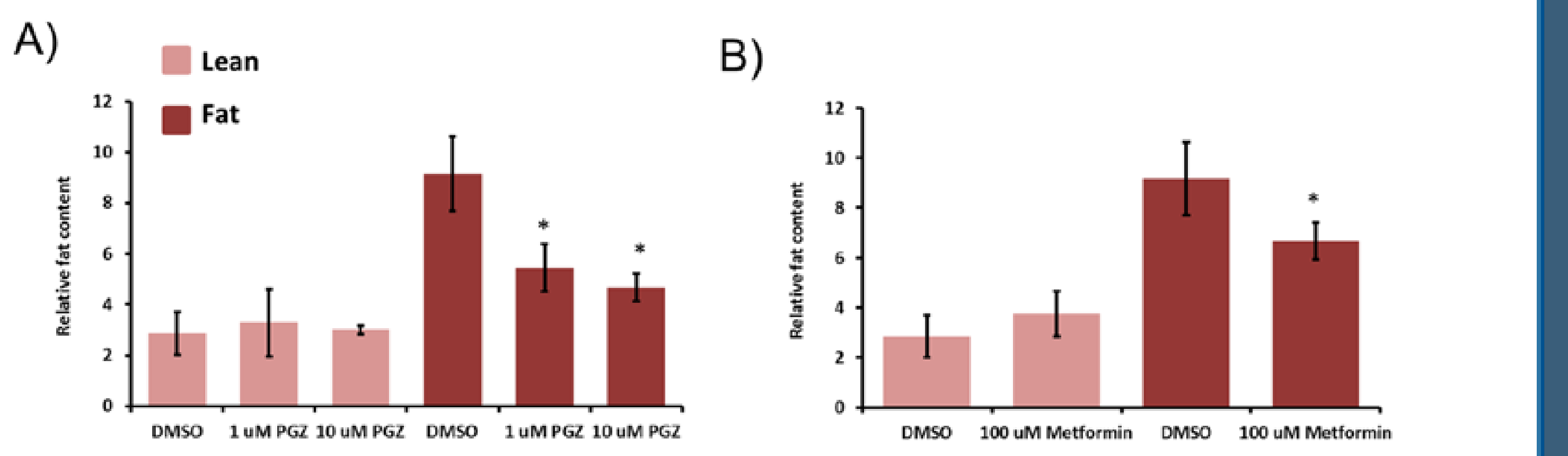

Figure 6 – Pioglitazone and Metformin can reduce steatosis in NAFLD model
PHH were cultured for 7 days in fat or lean conditions, before treatment with anti-steatotic compounds, A) pioglitazone and B) metformin. Cells were treated for 7 days in the continued presence/absence of fat. Fat content in all wells was analysed by Oil Red O staining and normalised to total protein content. Data is a mean ± SD (n =3), P < 0.05.

CONCLUSIONS

- Fat and lean culture conditions were developed for the culture of PHH for up to two weeks. Under fat conditions PHH continually accumulated fat and micro-steatotic lipid droplets were observed in tissues.
- Fat accumulation induces transcriptomic and proteomic changes that correlate with clinical observations from NAFLD patients, including upregulation of CYP2E1 expression and induction of fibrinogen production, with changes
- Fat loading reduced metabolic capacity of PHH, effecting numerous P450 enzymes
- Pioglitazone and metformin were demonstrated to reduce fat loading in the model.
- Model shows promise for applications including target discovery, ADME and toxicology.

REFERENCES

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