Tomasz Kostrzewski, Terri Cornforth, Sophie A Snow, Larissa Ouro-Gnao, Cliff Rowe, Emma M Large, David J Hughes,

CN Bio Innovations Limited, Welwyn Garden City AL7 3AX, United Kingdom

Author contributions: Kostrzewski T performed the majority of experiments and analysed the data; Cornforth T, Snow SA, Ouro-Gnao L performed experimental analysis; Kostrzewski T, Cornforth T, Rowe C, Large EM and Hughes DJ designed and coordinated the research; Kostrzewski T and Hughes DJ wrote the paper; all authors read and reviewed the paper.

Supported by Innovate UK (Technology Strategy Board) Advancing the Development and Application of Non-Animal Technologies Project: 3D cell culture model for studying Non-Alcoholic Fatty Liver Disease (NAFLD) - Ref: 131720.

Conflict-of-interest statement: At the time of this study all authors were employees of CN Bio Innovations Ltd.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Manuscript source: Invited manuscript

Correspondence to: David Hughes, DPhil, CN Bio Innovations Limited, BioPark, Broadwater Rd, Welwyn Garden City AL7 3AX, United Kingdom. david.hughes@cn-bio.com

Telephone: +44-1707-358739

Received: August 16, 2016
Peer-review started: August 17, 2016
First decision: October 11, 2016

Abstract

AIM
To develop a human in vitro model of non-alcoholic fatty liver disease (NAFLD), utilising primary hepatocytes cultured in a three-dimensional (3D) perfused platform.

METHODS
Fat and lean culture media were developed to directly investigate the effects of fat loading on primary hepatocytes cultured in a 3D perfused culture system. Oil Red O staining was used to measure fat loading in the hepatocytes and the consumption of free fatty acids (FFA) from culture medium was monitored. Hepatic functions, gene expression profiles and adipokine release were compared for cells cultured in fat and lean conditions. To determine if fat loading in the system could be modulated hepatocytes were treated with known anti-steatotic compounds.

RESULTS
Hepatocytes cultured in fat medium were found to accumulate three times more fat than lean cells and fat uptake was continuous over a 14-d culture. Fat loading of hepatocytes did not cause any hepatotoxicity and significantly increased albumin production. Numerous adipokines were expressed by fatty cells and genes associated with NAFLD and liver disease were upregulated including: Insulin-like growth factor-binding protein 1, fatty acid-binding protein 3 and CYP7A1. The metabolic activity of hepatocytes cultured in fatty conditions was found to be impaired and the activities of CYP3A4 and CYP2C9 were significantly reduced.

Three-dimensional perfused human in vitro model of non-alcoholic fatty liver disease

Tomasz Kostrzewski, Terri Cornforth, Sophie A Snow, Larissa Ouro-Gnao, Cliff Rowe, Emma M Large, David J Hughes
reduced, similar to observations made in NAFLD patients. The utility of the model for drug screening was demonstrated by measuring the effects of known anti-steatotic compounds. Hepatocytes, cultured under fatty conditions and treated with metformin, had a reduced cellular fat content compared to untreated controls and consumed less FFA from cell culture medium.

CONCLUSION
The 3D in vitro NAFLD model recapitulates many features of clinical NAFLD and is an ideal tool for analysing the efficacy of anti-steatotic compounds.

Key words: Non-alcoholic fatty liver disease; Liver disease; Three-dimensional cell culture; Organ-on-chip; Primary cell culture; Fatty liver; Hepatocytes

© The Author(s) 2017. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: We report the development of an in vitro, fully human, three-dimensional cell culture model of non-alcoholic fatty liver disease (NAFLD). The model recapitulates key features of clinical NAFLD, with primary human hepatocytes continuously loaded with fat, for up to 14 d of culture, without causing any hepatotoxicity. Fat loading caused transcriptomic, proteomic and metabolic changes to the hepatocytes, including reduced activity of CYP3A4 and CYP2C9 enzymes. Fat loading in the model could be modulated using known anti-steatotic drugs (e.g., metformin), demonstrating the utility of the model for screening the efficacy of novel anti-steatotic compounds.

INTRODUCTION
Due to the major increase in the prevalence of obesity, non-alcoholic fatty liver disease (NAFLD) is now considered to be among the most common liver diseases worldwide[1]. NAFLD is regarded as a broad spectrum of pathological conditions ranging from hepatic steatosis through to non-alcoholic steato-hepatitis (NASH) and liver fibrosis. Simple steatosis is a relatively benign state of liver injury, defined by the presence of fat in the liver which accounts for more than 5% of the liver's weight[2,2]. Fatty livers become vulnerable to further injury from oxidative stress, lipopapoptosis and inflammatory cytokines, which cause the development of NASH and cirrhosis[3]. Hepatic steatosis can often be a self-limiting state, but in up to 25% of cases the disease will progress to NASH and understanding why this transition takes place in certain individuals remains a key focus for research. The study of NAFLD also has important implications for drug metabolism and toxicity. Hepatocytes from steatotic livers often have altered metabolic capacity; with changes in expression of several cytochrome P450 (P450) enzymes and efflux transporter proteins[4,5]. Ultimately this can result in NAFLD patients having altered drug exposure and an elevated risk of adverse drug reactions[4,5].

Hepatic steatosis is characterised by the deposition of cytoplasmic triglycerides as macro- and/or micro-vesicular lipid vacuoles and this excessive accumulation of triglycerides arises from an imbalance in triglyceride acquisition and removal[6]. Investigating the molecular mechanisms that underlie steatosis and the hepatocellular consequences of triglyceride accumulation may be important in understanding the transition from steatosis to NASH and in the development of novel therapeutic interventions. Animal models (e.g., genetic models, diet-induced models) were first used to analyse the molecular basis of NAFLD and its development and many of these findings have been confirmed through clinical studies[6]. However, significant differences exist between humans and pre-clinical animal models in the way in which steatosis progresses, the inflammatory markers that are produced and the triglycerides that accumulate within hepatocytes[7]. Additionally, due to the complexity of these models it is difficult to analyse how the interplay between hepatic fat accumulation, environmental signals (oxidative stress and cytokines) and transcriptional regulation combine to cause the progression from steatosis to the more severe stages of NAFLD.

Some studies have also employed in vitro approaches to help elucidate the molecular mechanisms involved in disease progression, but compared to other liver diseases (e.g., viral hepatitis) only limited data has yet to be acquired using these approaches[8]. Various groups have analysed oleic acid-induced steatosis in hepatoma cell lines (e.g., HepG2 and HuH7) showing the accumulation of triglycerides reduces proliferation and transiently increases expression of inflammatory genes[9-12]. However, due to their immortalised nature, the capacity for drug metabolism of these cells is vastly reduced in comparison to primary cells[13]. They also lack expression of many key enzymes for lipid and fatty acid metabolism[14] and have altered intra- and extra-cellular signalling pathways[15].

Primary human hepatocyte cultures are a valuable model for analysing the mechanisms that underlie hepatic function as they most closely represent clinical conditions. The use of monolayer or sandwich in vitro cultures of hepatocytes has become common for metabolism and toxicology studies, but often these studies last for only a few days as hepatocytes lose metabolic capacity when cultured for longer[16]. Limited studies have looked at the development of steatosis with human hepatocytes in vitro. The combination of
Figure 1. LiverChip® three-dimensional perfused cell culture system. A: The LiverChip® plate contains 12 independent bioreactors for the 3D culture of hepatocytes; B: Hepatocytes form 3D micro-tissue structures in an array of channels in a collagen I-coated scaffold that is contained in each LiverChip® well; C: Schematic representation of how media flows through the channels of each LiverChip scaffold due to the action of a pneumatically operated pumps in the base of the plate. The speed and direction of flow can be adjusted using an electronic controller. 3D: Three-dimensional.

An 3D microtissue composed of cryopreserved human hepatocytes

Figure 1 LiverChip® three-dimensional perfused cell culture system. A: The LiverChip® plate contains 12 independent bioreactors for the 3D culture of hepatocytes; B: Hepatocytes form 3D micro-tissue structures in an array of channels in a collagen I-coated scaffold that is contained in each LiverChip® well; C: Schematic representation of how media flows through the channels of each LiverChip scaffold due to the action of a pneumatically operated pumps in the base of the plate. The speed and direction of flow can be adjusted using an electronic controller. 3D: Three-dimensional.

Several studies have suggested that intracellular fat accumulation in primary human hepatocytes undergoes changes in the presence of excess free fatty acids (FFA) for short periods of time, generally 12–48 h, in simple monolayer systems. Subsequently, only transient cellular changes were analysed and more long lasting effects of triglyceride accumulation were not studied.

Numerous three-dimensional (3D) cell culture models have now been developed that are proposed to better maintain the differentiated state of primary cells and provide more complex tissue organisation that was not previously possible in conventional 2D systems. Using organ-on-a-chip and microfluidic approaches, cell culture micro-environments can be created that recapitulate the mechanical forces, tissue-tissue interfaces, and spatiotemporal chemical gradients of living organs. Numerous advanced liver models have been described, which improve the long-term functionality of hepatocytes compared with 2D monocultures or simple 3D spheroid cultures.

Here, we introduce a cryopreserved primary human hepatocyte model of NAFLD in which the cells are cultured in 3D microtissues on an engineered scaffold that recapitulates the liver capillary bed under perfusion (Figure 1). The microtissues are cultured in the LiverChip® platform which contains twelve isolated bioreactors, each of which has an integrated micro-pump for controlling flow of culture medium through the tissue, creating an oxygen gradient across the tissue, like that observed in the in vivo liver sinusoid (Figure 1). Liver microtissues were cultured in lean or fat conditions to analyse the effect of triglyceride accumulation and the development of a NAFLD phenotype. Metabolic, transcriptome and phenotypic changes were measured in the system and these were found to mimic many of the changes observed in the clinical condition.

MATERIALS AND METHODS

Hepatocyte culture

Cryopreserved human hepatocytes were purchased from Life Technologies (Paisley, United Kingdom). Cells were thawed according to the instructions provided by the supplier. Viability was assessed using the trypan blue exclusion test and was > 85% for all lots. Cells were seeded into LiverChip® platforms (CN Bio Innovations, United Kingdom) (Figure 1), which were housed in a humidified cell culture incubator at 37 °C with 5% CO₂. The LiverChip® platforms consist of 12 individual bioreactors in which fluid is recirculated by a pneumatically driven micro-pump across a collagen-coated scaffold, which enables the formation of 3D microtissues in the channels of the scaffold (Figure 1). The platform is covered with a single loose lid, as per a standard microtiter plate enabling access to each of the 12 bioreactors for cell seeding, media change, and sampling.

Cells were seeded with flow in the downward direction through the scaffold for 8 h at a flow rate of 1.0 µL/s. Downward flow encourages cells to seed within the scaffold and form microtissues. Following cell attachment, the flow was changed to the upward direction and maintained at 1.0 µL/s for the remainder of the culture. Hepatocytes were seeded at a density of 6 × 10⁵ viable cells in 1.6 mL of medium per well. The cells were maintained in Williams’ E medium (WEM) containing primary hepatocyte thawing and plating supplements (Life Technologies, United States) for the first 24 h of culture. Thereafter, cells were cultured in lean or fat WEM, both of which contained physiologically relevant quantities of insulin (2 nmol/L) and glucose (5.5 mmol/L), as well as standard WEM supplements (0.5% Pen/Strep, 2 mmol GlutaMAX, 15 mmol/L HEPES, 6.25 ng/mL sodium selenite, 6.25 µg/mL transferrin, 1.25 mg/mL BSA, 5.35 µg/mL linoleic acid, 100 nmol/L dexamethasone). The fat media was further supplemented with 600 µmol/L FFA, containing a 2:1 mix of oleate acid and palmitate acid, conjugated to BSA. Complete media changes were performed on all wells every 48-72 h. Hepatocytes were also cultured in 2D in 96-well plates in lean and fat conditions. Post-thawing 5 × 10⁴ viable cells were seeded into each well of a collagen-coated 96-well plate. Cells were cultured in 150 µL media and treated equivalently to 3D-perfused samples.

Oil Red O staining and quantitation

Scaffolds containing microtissues were removed from plates, washed with PBS and fixed in 4% PFA (in PBS) for 15 min. Scaffolds were washed twice with
70% isopropanol for 5 min and then stained for 1 h in Oil Red O stain, which was a 3:2 mix of Oil Red O solution (Sigma Aldrich, United Kingdom) and dH₂O. Tissues were washed three times in dH₂O and twice in 70% isopropanol to remove non-specifically bound stain. Colour bright field images of stained scaffolds were taken using an inverted light microscope (Leica, United Kingdom). Oil Red O stain was removed from tissues by incubating them with 100% isopropanol for 15 min. The level of Oil Red O in the microtissues was quantified by analysing absorbance at 515 nm. Total cellular protein was determined for each sample following staining. Each scaffold was washed once in PBS and lysed in 0.1 mol NaOH + 2% SDS. Total cellular protein was measured with a Pierce BCA protein assay kit (Thermo Fisher, United Kingdom). The relative fat content of each sample was determined by normalising the level of Oil Red O, expressed as absorbance at 515 nm to the quantity of total protein.

Free fatty acid consumption
The consumption of free fatty acid from cell culture medium was determined using the Free Fatty Acid Quantification kit (Abcam, United Kingdom), following the manufacturers protocol. Free fatty acid consumption was calculated by comparing freshly prepared lean and fat media to conditioned media removed from plates at each media change.

Hepatocyte phenotyping
Albumin secretion was measured in culture supernatant using a human albumin enzyme-linked immunosorbent assay (Assay Pro, United States). Urea was quantified with a colorimetric assay kit (BioAssay Systems, United States) and lactate dehydrogenase (LDH) activity was measured using the CytoTox 96® Non-radioactive cytotoxicity assay (Promega, United Kingdom). Aspartate transaminase (AST) and alanine aminotransferase (ALT) clinical chemistry assays were performed at laboratories in the University of Pittsburgh Medical Center (Pittsburgh, United States). Total glutathione was quantified using the GSH-Glo™ Glutathione Assay (Promega, United Kingdom). Microtissues were lysed in 0.1 mol NaOH + 2% SDS. Total cellular protein was determined for each sample using a Pierce BCA protein assay kit (Thermo Fisher, United Kingdom). The consumption of free fatty acid from cell culture medium by liquid chromatography tandem mass spectrometry. Samples were separated using a Waters Acquity UPLC system with an ACE Excel C18-AR column and analysed on a Waters TQ-S Micro mass spectrometer against quantitative standard curves.

CYP3A assay
CYP3A activity was measured with the P450-Glo CYP3A4 assay with Luciferin-IPA (Promega, United Kingdom). A complete medium change to medium containing a 1000-fold dilution of Luciferin-IPA was performed and the plate incubated for 1 h at 37 °C, under flow. Samples from each well were placed in a 96-well assay plate and mixed 1:1 with luciferin detection reagent. The plate was incubated at room temperature for 20 min and luminescence measured relative to a standard curve of beetle luciferin potassium salt (Promega, United Kingdom).

RNA isolation and gene expression analysis
Total RNA was extracted from scaffolds cultured for 7 d in fat or lean conditions using TRIzol® Reagent (Ambion, United States) and a chloroform phase separation. RNA was precipitated from aqueous phase samples using 100% isopropanol and RNA pellets were resuspended in dH₂O. Reverse transcription and PCR were performed using RT® First Strand Kit and RT® Profiler PCR Arrays (Qiagen, United Kingdom) with 0.5 µg RNA analysed on each plate. Human Fatty Liver (PAHS-157Z) RT² Profiler™ PCR Arrays and Human Drug Metabolism (PAHS-002ZC-12) RT² Profiler™ PCR Arrays were used to analyse each sample. Samples were analysed using a Quantstudio 6 real time PCR system (Applied Biosystems, United Kingdom). Ct values from samples were compared and normalised to house-keeping gene expression. The fold-change in each transcript represented on the array plate was determined using RT² Profiler™ PCR Array Data Analysis (v.3.5) Web Portal (Qiagen, United Kingdom).

Adipokine proteome arrays
Cells were cultured in either fat or lean conditions for 7 d and conditioned media samples were analysed using the Proteome Profiler™ Human Adipokine array kits (R&D Systems, United States), following the manufactures protocol. Arrays were imaged using the LAS-3000 imaging system (Fujifilm, Japan) and images were analysed for pixel density using the ImageQuant software (GE Life Sciences, United Kingdom).

Liquid chromatography tandem mass spectrometry analysis for drug and metabolite quantification
To determine the activities of CYP -1A2, -2C9, -2D6 and -3A4 respectively, the conversion of Tacrine (5 µmol) to 1-hydroxytacrine, Diclofenac (90 µmol) to 4-hydroxydiclofenac, Bufuralol (10 µmol) to hydroxy-bufuralol and Midazolam (5 µmol) to 1-hydroxy-midazolam were analysed (compounds supplied by Sigma Aldrich, United Kingdom). A mixture of the four substrates was prepared at 1000-fold concentration in DMSO and added to microtissues during a full medium change. Cells were incubated for 2 h under standard culture conditions. Metabolites were quantified in cell culture medium by liquid chromatography tandem mass spectrometry. Samples were separated using a Waters Acquity UPLC system with an ACE Excel C18-AR column and analysed on a Waters TQ-S Micro mass spectrometer against quantitative standard curves.
**Exposure of hepatocytes to anti-steatotic compounds**

Microtissues were cultured for 7 d in lean or fat conditions, before dosing with anti-steatotic compounds. Pioglitazone hydrochloride and metformin hydrochloride (Sigma Aldrich, United Kingdom) were dissolved in DMSO, diluted in cell culture medium and added to microtissues during a full medium change. LiverChip® cultures were maintained in the same lean or fat conditions, in the presence of the compound, for a further 7 d.

**Statistical analysis**

All the experiments were performed with at least three replicates, with cells obtained from three different primary human hepatocyte donors, if not indicated otherwise. Values reported are mean ± SD, unless otherwise stated. Comparisons between groups were performed using Student’s t test. P values < 0.05 were considered statistically significant.

**RESULTS**

**In the presence of excess free fatty acid, hepatocytes can accumulate fat deposits without inducing cytotoxicity**

The 3D perfused in vitro model of NAFLD was created by seeding primary human hepatocytes into the LiverChip® platform and culturing the cells under fat and lean conditions. Both culture conditions contained physiologically relevant levels of glucose and insulin and fat media contained 600 µmol/L FFA. To assess the level of steatosis in microtissues, scaffolds were stained with Oil Red O, which demonstrated significant steatosis and triglyceride accumulation in samples cultured in fat media (Figure 2A). The absorbance of extracted Oil Red O from microtissues was spectrophotometrically determined and normalised to total cellular protein, to give a measure of relative fat content of each sample (Figure 2B). After 7 d of culture the fat media caused a significant increase in lipid accumulation and this further increased after 14 d (Figure 2B). Hepatocytes cultured in fat media also consumed more than four times the amount of FFA from the cell culture media, than equivalent cells in the lean condition (Figure 2C). Fatty acid consumption was determined by measuring changes in the concentration of FFA in the cell culture medium at each media change and comparing it to input media. The loss of FFA from the media was considered to be equivalent to the amount of FFA consumed by the cells.

Cryopreserved human hepatocyte donors have varying levels of basal fat, but all donors studied were observed to accumulate fat when cultured under fat conditions in the model (Supplementary Figure 1). Cell from the same donor were also cultured in 2D static monolayer cultures alongside the 3D-perfused platforms and were observed to produce significantly less albumin, had reduced expression of key metabolic genes and consumed lower quantities of FFA (Supplementary Figure 2). On this basis, the 3D perfused platform was selected for the in vitro NAFLD model.

Overloading hepatic cells with FFA has previously been observed to cause cytotoxic effects12,17, therefore we analysed the NAFLD model for cell death and liver-specific functions. Under fat conditions hepatocytes were fully viable and fat loading significantly increased their secretion of albumin (Figure 2D), whilst AST and alanine transaminase (ALT) levels were not affected (Figure 2E). LDH release, urea genesis and mitochondrial activity, as measured by WST-1 and cellular glutathione levels, were also not affected by fat loading (Supplementary Figure 3). Taken together this demonstrates hepatocytes cultured in the 3D perfused platform under fat conditions are fully viable, functional and continually become more steatotic.

**Fat loading induces transcriptional and proteomic changes in hepatocytes**

The development of NAFLD and hepatic steatosis are associated with a range of transcriptomic and proteomic changes, particularly in genes associated with inflammation, cholesterol/lipid metabolism and insulin signalling31. We therefore explored whether our 3D in vitro NAFLD model recapitulated the changes observed in vivo. We analysed the expression of key genes associated with fatty liver and a number of these were observed to be upregulated in cells cultured in fatty conditions, including CYP2E1, IGF1, PDK4 and CYP7A1 (Figure 3) (Supplementary Table 1). In addition we explored transcriptional changes in genes associated with hepatic metabolism and found upregulation of several P450 genes, as well as the gene for alcohol dehydrogenase C1 in fat loaded samples (Supplementary Figure 4, Supplementary Table 2). In addition to exploring changes at the transcriptional level we analysed the secretion of key adipokines from cells cultured in fat and lean conditions. Fat loaded hepatocytes secreted a range of adipokines, and in comparison to lean cultured cells, produced increased quantities of pro-inflammatory markers IL-8 and MIF; as well as adipokines associated with fibrosis and wound healing, including fibrinogen and TIMP-1 (Figure 4). The changes observed at the transcriptional and proteomic level in our 3D in vitro model mimic many of the changes commonly associated with NAFLD/steatosis5,27.

**Metabolic capacity of hepatocytes is affected by fat loading**

As the transcription of numerous metabolic genes were altered in the NAFLD model, we investigated how fat loading affected the metabolic capacity of the hepatocytes. The 3D perfused platform has previously been shown to maintain the metabolic capacity of human hepatocytes for extended periods of culture25. Initially, we used the CYP3A-Glo assay and found for
multiple hepatocyte donors fat loading for seven days caused a significant reduction in P450 activity (Figure 5A). The different donors had varying P450 activity, but when each donor was cultured in the fat media its CYP3A activity was reduced by ≥ 40% (Figure 5A). We explored the activity of CYP1A2, CYP2C9, CYP2D6 and CYP3A4, in more detail by measuring their ability to metabolise probe compounds. Fat loaded hepatocytes
had significantly reduced CYP3A4 and CYP2C9 activity when compared to cells cultured in lean media (Figure 5B). CYP3A4 activity was reduced by almost half and CYP2C9 activity was reduced by 30%, however no differences were observed for CYP1A2 and CYP2D6 activity (Figure 5). These observations align with those of previous studies involving human hepatocytes and clinical samples from NAFLD patients, which for example have shown that CYP3A4 activity decreases with disease progression\cite{28,29}.

**Steatosis can be reduced using current NAFLD therapies**

We explored whether the level of fat loading into hepatocytes could be modulated using known anti-steatotic compounds. Both pioglitazone and metformin have been shown to have anti-steatotic effects in vivo, ultimately leading to improved clinical outcomes\cite{30,31}. Hepatocytes were cultured for one week in lean or fat conditions and then continued for a second week in the same conditions, but in the presence of pioglitazone, metformin or vehicle control (Figure 6).

Both pioglitazone (Figure 6A) and metformin (Figure 6B) significantly reduced fat loading of hepatocytes, whilst neither affected hepatic function, as determined by albumin production (Figure 6C). Physiologically relevant concentrations of both drugs, which were representative of therapeutic doses achievable in vivo, were used to test in the model. Pioglitazone had the most significant effect on hepatic fat accumulation, reducing the relative fat content by 50% when dosed at 10 µmol (Figure 6A), however we did not observe pioglitazone to affect inflammation or fibrosis markers in the model; the amount of IL-8 and fibrinogen expressed by dosed cells was the same as control cells (data not shown). Neither compound affected the cells cultured in lean media, which accumulate only small quantities of fat. The two compounds were demonstrated to have differing modes of action, as only metformin was observed to affect the consumption of FFA from the cell culture medium, with treated cells consuming up to 20% less FFA (Figure 6D). This data demonstrates the utility of the 3D in vitro NAFLD model for drug screening and its potential utility in discovering compounds that can prevent or reverse hepatic steatosis.
DISCUSSION

The utilisation of advanced or organ-on-a-chip type in vitro culture systems that more closely mimic the microenvironment of the liver are now established as a method for maintaining the functions of primary human hepatocytes in vitro and preventing rapid dedifferentiation [20,21]. We have utilised a 3D perfused platform to develop an all human in vitro model of NAFLD. The main hallmark of NAFLD is intrahepatic triglyceride accumulation (steatosis), which initially leads to a benign condition, but with the correct additional stimuli can progress to more advanced conditions of steatohepatitis and fibrosis.

We cultured primary human hepatocytes as 3D microtissues and exposed them to 600 µmol FFA, which caused significant triglyceride accumulation and lead to a range of phenotypic changes similar to those observed in NAFLD patients. Our model utilised a combination of palmitic and oleic acids, which are common dietary long-chain FFAs found to accumulate in excess in human livers with steatosis [32,33]. When cultured in vitro, hepatocytes are also known to accumulate fat when exposed to high levels of glucose [34]. Therefore the media conditions used in this model were selected to have lower, more physiologically relevant, glucose and insulin concentrations when compared to standard hepatocyte culture media [35]. This media composition...
allowed us to study the specific effect of triglyceride accumulation on hepatocytes.

In fat media conditions cells were observed to continually accumulate intracellular triglycerides without showing signs of cytotoxicity. However, it is well established that when lipids accumulate in non-adipose tissues they can enter non-oxidative, deleterious pathways, potentially leading to cell injury and death. Previous studies have shown that fat loading of hepatocytes cultured in vitro can cause cytotoxicity, particularly when high concentrations of saturated fatty acids are present\(^\text{17}\). The concentration of FFA and ratio of saturated to unsaturated FFAs (1:2) used in our model were selected as these had previously been previously shown to not cause hepatotoxicity, whilst inducing significant levels of intracellular triglyceride accumulation\(^\text{9,11,17}\).

We observed a four-fold increase in hepatocyte triglyceride accumulation between fat and lean cultured cells, which is a similar increase to that previously reported for short term 2D cultures of hepatocytes cultured with FFAs\(^\text{12,17}\) and for hepatocytes isolated from steatotic human livers compared to those obtained from healthy, non-steatotic livers\(^\text{36}\). Under fat conditions cells could maintain hepatic functions in the 3D culture system for up to two weeks. To our knowledge this is significantly longer than all previous in vitro steatosis studies, which have either analysed freshly isolated primary hepatocytes or cultured cells with FFA for less than 5 d. This allowed the analysis of longer term, progressive effects of exposing hepatocytes to excess FFA, rather than simply looking at the transient changes that may occur in the cells when they are first exposed. Interestingly, throughout the 14-d culture, albumin production was significantly higher from cells cultured with excess FFA. Similar observations have been made in vivo, where increased serum albumin concentrations have been associated with insulin resistance, diabetes and over nutrition\(^\text{37,38}\). However, such findings have not always been consistent, and some studies of NAFLD patient subsets suggest that serum albumin concentrations are not affected by the development of disease\(^\text{39}\). It may be that early stages of steatosis and intrahepatic fat accumulation lead to increased albumin production, which is balanced out in later stages of NAFLD when inflammation and fibrosis begin to more severely negatively impact hepatic function.

To more fully appreciate the other phenotypic changes that occur in hepatocytes during the accumulation of triglycerides we analysed both transcriptomic and proteomic changes in the model. Numerous transcriptional changes were observed after exposing the microtissues to FFA in the 3D-perfused platform, including a five-fold increase in CYP2E1 expression. Higher hepatic CYP2E1 expression and activity have been frequently observed in the context of NAFLD and obesity\(^\text{30,41}\). It has been suggested that this increase can result in reactive oxygen species overproduction which can promote NAFLD pathogenesis\(^\text{40,42}\). It should be noted that we only measured transcriptional changes in CYP2E1 and it is known that these do not always correlate well with enzyme activity\(^\text{28}\) and future studies should ascertain how CYP2E1 enzyme activity is affected by fat loading in the model. CYP7A1, a key gene in cholesterol metabolism, was also upregulated in the model. This upregulation has previously been observed in NAFLD/NASH patients, who have increased free cholesterol levels and the dysregulation of cholesterol metabolism has been correlated with disease severity\(^\text{43}\). Further genes associated with insulin resistance and lipid metabolism, such as IGF1, PDK4 and FABP-1 were also upregulated in fat loaded hepatocytes. IGF1 has been identified as an early marker for metabolic liver disease\(^\text{18}\), whilst PDK4 has been found to be directly linked to hepatic insulin resistance\(^\text{45}\). It is important to note that steatosis is the first stage in NAFLD pathogenesis and is not associated with gross hepatic functional changes. Therefore as expected we did not observe large numbers of transcriptional changes that have been reported for more advanced stages of NAFLD and NASH\(^\text{46}\).

When we analysed the proteins secreted by the hepatocytes, the accumulation of fat also affected the adipokines released by the cells, influencing the secretion of proteins associated with fibrosis and liver injury (fibrinogen, TIMP-1) as well as inflammatory markers (IL-8 and MIF). It has been widely reported that NAFLD/NASH patients have elevated serum levels of pro-inflammatory cytokines, in particular IL-8\(^\text{47}\) and previously palmitic acid has been shown to stimulate IL-8 expression in cultured human hepatocytes\(^\text{48}\). Increased MIF expression is associated with NAFLD development in vivo, with liver biopsies of NAFLD patients showing higher expression of MIF compared to healthy controls\(^\text{49}\). Animal studies have also shown an association between high fat diets, insulin resistance and the plasma level of MIF\(^\text{49}\). Fibrinogen expression is also associated with fatty liver disease as its expression increases with liver stress and damage\(^\text{50}\).

Despite these changes other inflammatory cytokines associated with NAFLD, such as IL-1 and IL-6, were not observed to be secreted by fat loaded cells in the model. This is likely due to a lack of non-parenchymal cells in our model which are the primary sources of inflammatory cytokines during NAFLD development, in particular IL-6 is highly expressed by Kupffer cells\(^\text{51}\). The adipokines observed to change in the model including IL-8, fibrinogen, and MIF are known to be expressed by hepatocytes and are all associated with a NAFLD phenotype\(^\text{18,47,48}\).

Hepatic steatosis is known to affect the metabolic capacity of hepatocytes, by affecting the expression and activity of P450 enzymes and cell membrane transporters\(^\text{5,29}\). In our NAFLD model the metabolic rates of both CYP3A4 and CYP2C9 were reduced, but the gene expression of both enzymes was not affected. Similar changes in P450 activity were previously
Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of chronic liver diseases, ranging from hepatic steatosis or fatty liver, non-alcoholic steatohepatitis, liver fibrosis and liver cirrhosis. It is estimated that more than 1 billion people worldwide are now affected by some form of NAFLD, but currently no specific treatments options are available and the only curative therapy option available is liver transplantation. To understand the molecular basis of this disease in more detail better experimental models are required that fully recapitulate the phenotype of the human disease.

Research frontiers
Previous in vitro studies of NAFLD have generally involved in the use of hepatoma cell lines or the exposure of primary hepatocytes to triglycerides for short periods of time (<48 h) and therefore do not capture the complexity of the disease.

Innovations and breakthroughs
The authors have generated a novel three-dimensional (3D) perfused, all human NAFLD model to be a tool for screening drugs as NAFLD therapeutics by analysing the activities of two known anti-steatotic compounds, pioglitazone and metformin. When dosed in the model both compounds reduced the level of triglyceride accumulation in the fat loaded cells, but appeared to function by different mechanisms. Pioglitazone has previously been reported to reduce hepatic steatosis in animal models of NAFLD and shown to have some benefit to NAFLD patients in clinical trials. The direct effect of pioglitazone on hepatocytes, which can reduce triglyceride accumulation, is by its ability to alter lipid metabolism and mediate lipoprotein lipase expression through the activation of PPARγ. Pioglitazone is also known to have indirect effects on hepatic steatosis by influencing the release of FFA from adipose tissue. We observed its ability to reduce triglyceride accumulation, but it did not affect the consumption of FFA from the cell culture media. In contrast, metformin was able to reduce FFA consumption from the culture medium, leading to reduced intrahepatic triglyceride concentration. This finding was previously reported for metformin treatment of murine models of NAFLD and metformin has been found to decrease the expression of lipid-droplet associated proteins such as ADRP and inflammatory markers, including TNFα.

Our results demonstrate that the prolonged culture of human hepatocytes in vitro, under fat conditions mimics many of the effects observed in clinical NAFLD. The 3D perfused nature of the model allows for sustained culture of hepatocytes in vitro, allowing prolonged effects of FFA accumulation to be explored. This model is therefore well suited to exploring the molecular mechanisms that underlie steatosis and has the potential to be used as a tool for drug screening. Using the 3D-perfused platform multicellular liver models (e.g., hepatocytes and Kupffer cells) have already been developed  and could potentially be combined with our steatosis model to create a further more advanced model of NAFLD/NASH.

ACKNOWLEDGMENTS
The authors thank Dr Alan Wells and Dr Amanda Clark (University of Pittsburgh) for performing clinical chemistry assays for ALT and AST. Mass Spectrometry was performed by an independent contract research organisation Xenogenesis Ltd (Nottingham, United Kingdom). Adipokine arrays were performed by contract research organisation Labstract Ltd (Cambridge, United Kingdom).

COMMENTS
Background
Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of chronic liver diseases, ranging from hepatic steatosis or fatty liver, non-alcoholic steatohepatitis, liver fibrosis and liver cirrhosis. It is estimated that more than 1 billion people worldwide are now affected by some form of NAFLD, but currently no specific treatments options are available and the only curative therapy option available is liver transplantation. To understand the molecular basis of this disease in more detail better experimental models are required that fully recapitulate the phenotype of the human disease.

Research frontiers
Previous in vitro studies of NAFLD have generally involved in the use of hepatoma cell lines or the exposure of primary hepatocytes to triglycerides for short periods of time (<48 h) and therefore do not capture the complexity of the disease.

Innovations and breakthroughs
The authors have generated a novel three-dimensional (3D) perfused, all human NAFLD model to be a tool for screening drugs as NAFLD therapeutics by analysing the activities of two known anti-steatotic compounds, pioglitazone and metformin. When dosed in the model both compounds reduced the level of triglyceride accumulation in the fat loaded cells, but appeared to function by different mechanisms. Pioglitazone has previously been reported to reduce hepatic steatosis in animal models of NAFLD and shown to have some benefit to NAFLD patients in clinical trials. The direct effect of pioglitazone on hepatocytes, which can reduce triglyceride accumulation, is by its ability to alter lipid metabolism and mediate lipoprotein lipase expression through the activation of PPARγ. Pioglitazone is also known to have indirect effects on hepatic steatosis by influencing the release of FFA from adipose tissue. We observed its ability to reduce triglyceride accumulation, but it did not affect the consumption of FFA from the cell culture media. In contrast, metformin was able to reduce FFA consumption from the culture medium, leading to reduced intrahepatic triglyceride concentration. This finding was previously reported for metformin treatment of murine models of NAFLD and metformin has been found to decrease the expression of lipid-droplet associated proteins such as ADRP and inflammatory markers, including TNFα.

Our results demonstrate that the prolonged culture of human hepatocytes in vitro, under fat conditions mimics many of the effects observed in clinical NAFLD. The 3D perfused nature of the model allows for sustained culture of hepatocytes in vitro, allowing prolonged effects of FFA accumulation to be explored. This model is therefore well suited to exploring the molecular mechanisms that underlie steatosis and has the potential to be used as a tool for drug screening. Using the 3D-perfused platform multicellular liver models (e.g., hepatocytes and Kupffer cells) have already been developed and could potentially be combined with our steatosis model to create a further more advanced model of NAFLD/NASH.

ACKNOWLEDGMENTS
The authors thank Dr Alan Wells and Dr Amanda Clark (University of Pittsburgh) for performing clinical chemistry assays for ALT and AST. Mass Spectrometry was performed by an independent contract research organisation Xenogenesis Ltd (Nottingham, United Kingdom). Adipokine arrays were performed by contract research organisation Labstract Ltd (Cambridge, United Kingdom).

COMMENTS
Background
Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of chronic liver diseases, ranging from hepatic steatosis or fatty liver, non-alcoholic steatohepatitis, liver fibrosis and liver cirrhosis. It is estimated that more than 1 billion people worldwide are now affected by some form of NAFLD, but currently no specific treatments options are available and the only curative therapy option available is liver transplantation. To understand the molecular basis of this disease in more detail better experimental models are required that fully recapitulate the phenotype of the human disease.

Research frontiers
Previous in vitro studies of NAFLD have generally involved in the use of hepatoma cell lines or the exposure of primary hepatocytes to triglycerides for short periods of time (<48 h) and therefore do not capture the complexity of the disease.

Innovations and breakthroughs
The authors have generated a novel three-dimensional (3D) perfused, all human NAFLD model to be a tool for screening drugs as NAFLD therapeutics by analysing the activities of two known anti-steatotic compounds, pioglitazone and metformin. When dosed in the model both compounds reduced the level of triglyceride accumulation in the fat loaded cells, but appeared to function by different mechanisms. Pioglitazone has previously been reported to reduce hepatic steatosis in animal models of NAFLD and shown to have some benefit to NAFLD patients in clinical trials. The direct effect of pioglitazone on hepatocytes, which can reduce triglyceride accumulation, is by its ability to alter lipid metabolism and mediate lipoprotein lipase expression through the activation of PPARγ. Pioglitazone is also known to have indirect effects on hepatic steatosis by influencing the release of FFA from adipose tissue. We observed its ability to reduce triglyceride accumulation, but it did not affect the consumption of FFA from the cell culture media. In contrast, metformin was able to reduce FFA consumption from the culture medium, leading to reduced intrahepatic triglyceride concentration. This finding was previously reported for metformin treatment of murine models of NAFLD and metformin has been found to decrease the expression of lipid-droplet associated proteins such as ADRP and inflammatory markers, including TNFα.

Our results demonstrate that the prolonged culture of human hepatocytes in vitro, under fat conditions mimics many of the effects observed in clinical NAFLD. The 3D perfused nature of the model allows for sustained culture of hepatocytes in vitro, allowing prolonged effects of FFA accumulation to be explored. This model is therefore well suited to exploring the molecular mechanisms that underlie steatosis and has the potential to be used as a tool for drug screening. Using the 3D-perfused platform multicellular liver models (e.g., hepatocytes and Kupffer cells) have already been developed and could potentially be combined with our steatosis model to create a further more advanced model of NAFLD/NASH.

ACKNOWLEDGMENTS
The authors thank Dr Alan Wells and Dr Amanda Clark (University of Pittsburgh) for performing clinical chemistry assays for ALT and AST. Mass Spectrometry was performed by an independent contract research organisation Xenogenesis Ltd (Nottingham, United Kingdom). Adipokine arrays were performed by contract research organisation Labstract Ltd (Cambridge, United Kingdom).
REFERENCES

1. Moore JB. Non-alcoholic fatty liver disease: the hepatic consequence of obesity and the metabolic syndrome. *Proc Nutr Soc* 2010; 69: 211-220 [PMID: 20158939 DOI: 10.1017/S0029665110000300]

2. Than NN, Newsome PN. A concise review of non-alcoholic fatty liver disease. *Atherosclerosis* 2015; 239: 192-202 [PMID: 25617860 DOI: 10.1016/j.atherosclerosis.2015.01.001]

3. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 2004; 114: 147-152 [PMID: 15254578 DOI: 10.1172/JCI22422]

4. Donato MT, Lahoz A, Jiménez N, Pérez G, Serrallada M, Mir J, Castell JV, Gómez-Lechón MJ. Potential impact of steatosis on cytotoxicity of 3P430 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab Dispos* 2006; 34: 1556-1562 [PMID: 16763015 DOI: 10.1124/dmd.106.009670]

5. Lake AD, Novak P, Fisher CD, Jackson JP, Hardwick RN, Billheimer DD, Klimkewi WT, Cherrington NJ. Analysis of global and absorption, distribution, metabolism, and elimination gene expression in the progressive stages of human nonalcoholic fatty liver disease. *Drug Metab Dispos* 2011; 39: 1954-1960 [PMID: 21737566 DOI: 10.1124/dmd.111.045092]

6. Sanches SC, Ramalho LN, Augusto MJ, da Silva DM, Ramalho FS. Nonalcoholic Steatohepatitis: A Search for Factual Animal Models. *Biomed Res Int* 2015; 2015: 574832 [PMID: 26064924 DOI: 10.1155/2015/574832]

7. Willebroords J, Pereira IV, Maes M, Crespo Yanguas S, Colle I, Van Den Bossche B, Da Silva TC, de Oliveira CP, Andrade I, Van Den Bossche B, Da Silva TC, de Oliveira CP, Andrade I. Hepatocyte-derived reporter cells for study the effects of intracellular fat accumulation and subsequent 3D cell culture to organs-on-chips. *Trends Cell Biol* 2011; 21: 745-754 [PMID: 22033488 DOI: 10.1016/j.tcb.2011.09.005]

8. Ebrahimkhani MR, Neiman JA, Raredon MS, Hughes DJ, Griffith LG. Bioreactor technologies to support liver function in vitro. *Adv Drug Deliv Rev* 2014; 69-70: 132-157 [PMID: 24670703 DOI: 10.1016/j.addr.2014.02.011]

9. Lin C, Ballinger KR, Khatri SR. The application of engineered liver tissues for novel drug discovery. *Expert Opin Drug Discov* 2015; 10: 519-540 [PMID: 25840592 DOI: 10.1517/17440494.20.1.503224]

10. Esch EW, Bahnisi A, Huh D. Organs-on-chips at the frontiers of drug discovery. *Nat Rev Drug Discov* 2015; 14: 248-260 [PMID: 25792263 DOI: 10.1038/nrd4539]

11. Sarkar U, Kheifets LA, D’Angio GJ. Experimental non-alcoholic fatty liver disease: current status and a glimpse of the future. *World J Gastroenterol* 2015; 21: 1091-1099 [PMID: 25926341 DOI: 10.1124/did.115.063495]

12. Domansky K, Inman W, Serdy J, Dash A, Lim MH, Griffith LG. Perfusion multiwell plate for 3D liver tissue engineering. *Lab Chip* 2010; 10: 51-58 [PMID: 20024050 DOI: 10.1039/b913221j]

13. Vivas A, Sale-LeFort S, Araybay-Fabre C, Ngo R, Penarier G, Bremond M, Mollerin P, Gallais JF, Fabre G, Klieber S. Morphological behaviour and metabolic capacity of cryopreserved human primary hepatocytes cultivated in a perfused multiwell device. *Xenobiotica* 2015; 45: 29-44 [PMID: 25068923 DOI: 10.3109/09534734.2014.944612]

14. Fitzpatrick E, Dhawan A. Noninvasive biomarkers in non-alcoholic fatty liver disease: current status and a glimpse of the future. *J Clin Invest* 2014; 124: 1085-1086 [PMID: 25152587 DOI: 10.1172/JCI23110851]

15. Wruck W, Kashofer K, Rehaman S, Daskalaki A, Berg D, Gratk S, Jozefczuk J, Dresw K, Pandey V, Regenbok C, Wiecresting K, Turano P, Komurc D, Loebrach H, Westerhoff HV, Adjaye J. Multi-omic profiles of human non-alcoholic fatty liver disease tissue highlight inheritable phenotypes. *Sci Data* 2015; 2: 150068 [PMID: 26646939 DOI: 10.1038/sdata.2015.68]

16. Fisher CD, Lickett AJ, Augustine LM, Ranger-Moore J, Jackson JP, Ferguson SS, Cherrington NJ. Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos* 2009; 37: 2087-2094 [PMID: 19651758 DOI: 10.1021/dm9002746]

17. Donato MT, Lahoz A, Jiménez N, Serrallada M, Mir J, Castell JV, Gómez-Lechón MJ. Effect of steatois on drug-metabolizing capability of primary human hepatocytes. *Toxicol In Vitro* 2017; 21: 271-276 [PMID: 16950596 DOI: 10.1016/j.tiv.2006.07.008]

18. Tan HZ, Yang SQ, Chuckaree C, Kuhafja G, Ronnet G, Diehl AM. Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med* 2000; 6: 98-1003 [PMID: 10973319 DOI: 10.1038/sdata.2015.68]

19. Aithal GP, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, Austin AS, Freeman JG, Morgan L, Webber J. Randomized, placebo-controlled trial of pioglitazone in nonalcoholic hepatic steatohepatitis. *Gastroenterology* 2008; 135: 1176-1184 [PMID: 18718471 DOI: 10.1053/j.gastro.2008.06.047]

20. Pur I, Baille RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007; 46: 1001-1009
Kostrewski T et al. Human in vitro model of NAFLD

insulin resistance induced diabetes. PLoS One 2013; 8: e71997 [PMID: 23940800 DOI: 10.1371/journal.pone.0071997]

Anstee QM, Day CP. The genetics of NAFLD. Nat Rev Gastroenterol Hepatol 2013; 10: 645-655 [PMID: 24061205 DOI: 10.1038/nrgastro.2013.182]

Braunersreuther V, Viviani GL, Mach F, Monteucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. World J Gastroenterol 2012; 18: 727-735 [PMID: 22371632 DOI: 10.3748/wjg.v18.i8.727]

Akyildiz M, Gunsar F, Nart D, Sahin O, Yilmaz F, Akay S, Erooz G, Karasu I, Ilter T, Batur Y, Berdeli A, Akareka U. Macrophage migration inhibitory factor expression and MIF gene -173 G/C polymorphism in nonalcoholic fatty liver disease. Eur J Gastroenterol Hepatol 2010; 22: 192-198 [PMID: 19829123 DOI: 10.1097/MEG.0b013e32835a9596]

Morrison MC, Kleemann R. Role of Macrophage Migration Inhibitory Factor in Obesity, Insulin Resistance, Type 2 Diabetes, and Associated Hepatic Co-Morbidities: A Comprehensive Review of Human and Rodent Studies. Front Immunol 2015; 6: 308 [PMID: 26124760 DOI: 10.3389/fimmu.2015.00308]

Paschos P, Paletas K. Non-alcoholic fatty liver disease and metabolic syndrome. Hippokratia 2009; 13: 9-19 [PMID: 19240815]

Schmidt-Arras D, Rose-John S. IL-6 pathway in the liver: from physiopathology to therapy. J Hepatol 2016; 64: 1403-1415 [PMID: 26867490 DOI: 10.1016/j.jhep.2016.02.004]

Hardy T, Anstee QM, Day CP. Nonalcoholic fatty liver disease: new treatments. Curr Opin Gastroenterol 2015; 31: 175-183 [PMID: 25774446 DOI: 10.1097/MOG.0000000000000175]

Qin S, Liu T, Kamanna VS, Kashyap ML. Pioglitazone stimulates apolipoprotein A-I production without affecting HDL removal in HepG2 cells: involvement of PPAR-alpha. Arterioscler Thromb Vasc Biol 2007; 27: 2428-2434 [PMID: 17872455 DOI: 10.1161/ATVBAHA.107.150193]

Sakamoto J, Kimura H, Moriyama S, Odaka H, Momose Y, Sugiyama Y, Sawada H. Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. Biochem Biophys Res Commun 2009; 380: 704-711 [PMID: 11095972 DOI: 10.1016/j.bbrc.2009.07.119]

Nagashima K, Lopez C, Donovan D, Ngai C, Fontanze N, Bensadoun A, Fruchtach-Najib J, Holleran S, Cohn JS, Ramakrishnan R, Ginsberg HN. Effects of the PPARgamma agonist pioglitazone on lipoprotein metabolism in patients with type 2 diabetes mellitus. J Clin Invest 2005; 115: 1323-1332 [PMID: 15841215 DOI: 10.1172/JCI23219]

Sciple RK, Chatterjee VK, O’Rahilly S. PPAR gamma and human metabolic disease. J Clin Investig 2006; 116: 581-589 [PMID: 16511590 DOI: 10.1172/JCI28003]

Liu F, Wang C, Zhang L, Xu Y, Jang L, Gu Y, Cao X, Zhao X, Ye J, Li Q. Metformin prevents hepatic steatosis by regulating the expression of adipose differentiation-related protein. Int J Mol Med 2014; 33: 51-58 [DOI: 10.3892/ijmm.2013.1560]

Federico A, Zulli C, de Sio I, Del Prete A, Dallio M, Masarone M, Loguerico C. Focus on emerging drugs for the treatment of patients with non-alcoholic fatty liver disease. World J Gastroenterol 2014; 20: 16841-16857 [PMID: 25492998 DOI: 10.3748/wjg.v20.i45.16841]
