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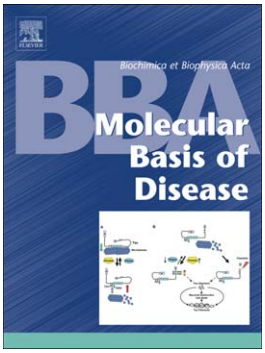

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Title: **Lipotoxicity and steatohepatitis in an overfed mouse model for non-alcoholic fatty liver disease.**

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Abstract

The major risk factors for non-alcoholic fatty liver disease (NAFLD) are obesity, insulin resistance and dyslipidemia. The cause for progression from the steatosis stage to the inflammatory condition (non-alcoholic steatohepatitis (NASH)) remains elusive at present,. Aim of this study was to test whether the different stages of NAFLD as well as the associated metabolic abnormalities can be recreated in time in an overfed mouse model and study the mechanisms underlying the transition from steatosis to NASH.

Male C57Bl/6J mice were subjected to continuous intragastric overfeeding with a high-fat liquid diet (HFLD) for different time periods. Mice fed a solid high-fat diet (HFD) *ad libitum* served as controls. Liver histology and metabolic characteristics of liver, white adipose tisue (WAT) and plasma were studied.

Both HFD-fed and HFLD-overfed mice initially developed liver steatosis, but only the latter progressed in time to NASH. NASH coïncided with obesity, hyperinsulinemia, loss of liver glycogen and hepatic endoplasmatic reticulum stress. Peroxisome proliferator-activated receptor γ (*Pparγ*), fibroblast growth factor 21 (*Fgf21*), fatty acid binding protein (*Fabp*) and fatty acid translocase (CD36) were induced exclusively in the livers of the HFLD-overfed mice.. Inflammation, reduced adiponectin expression and altered expression of genes that influence adipogenic capacity were only observed in WAT of HFLD-overfed mice.

In conclusion: this dietary mouse model displays the different stages and the metabolic settings often found in human NAFLD. Lipotoxicity due to compromised adipose tissue function is likely associated with the progression to NASH, but whether this is cause or consequence remains to be established.

Keywords

Non-alcoholic fatty liver disease; steatohepatitis; liver; overnutrition; high-fat diet; endoplasmatic reticulum stress; adipose tissue.

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1. Introduction

.If hepatic import and synthesis of triglycerides exceeds hepatic triglyceride export and oxidation, triglycerides accumulate in the hepatocytes (steatosis stage of non-alcoholic fatty liver disease (NAFLD)). Several genetic and dietary animal models have been used to study the progression from steatosis to the stage of steatohepatitis or inflamed fatty liver (NASH; reviewed a.o. by Larter and Yeh [1]). However, the mechanisms underlying this transition have remained largely obscure due to a lack of animal models that simulate the etiology of the human disease. The severity of steatosis is significantly associated with NASH[2,3] and only part of the patients progress to steatohepatitis. Therefore it has been hypothesized that a 'double hit' is required for the development of NASH[4]: the first producing steatosis and sensitizing the liver for the second hit (*i.e* oxidative stress[5] or (endotoxic) cytokines[6]. However, an obligatory role for oxidative stress in the progression to NASH could not been shown[7], which led to the alternative "lipotoxity" hypothesis (reviewed by Neuschwander-Tetri[8]). Lipotoxicity attributes cellular injury and death to fatty acids and/or their metabolites[9,10]. Although intracellular fatty acid concentration is quite constant in hepatocytes[11], the flux of fatty acids through the liver is increased in NASH patients[12,13]. This may be caused either through increased supply by excessive or inappropriate adipocyte lypolysis[14] or excessive de novo lipogenesis[15]. Combined with an imbalanced fatty acid oxidation, lipotoxicity may occur.

NAFLD is a highly prevalent disease, the worldwide incidence of which has increased significantly concomitant with the increase in the number of obese people [16]. The most common cause of obesity in man is a chronic intake of a hypercaloric diet rich in (saturated) fats. Deng et al. [17] developed a NAFLD model in which mice were continuously overfed a high-fat diet via a gastrostomy tube for 9 weeks. These mice became obese, insulin-resistant and diabetic, and developed steatohepatitis. In the present study, we used a similar mouse model in a longitudinal study to follow the development of liver pathology in time and to characterize the mechanisms underlying the progression from steatosis to NASH.

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2. Materials and methods

2.1 Surgical procedure and dietary regimen

Ten-weeks old, male C57Bl/6J mice (Harlan NL) had a catheter inserted into the forestomach via a purse string suture. The catheter was attached to a 20G swivel to allow continuous infusion of feed and free movement of the mice (all infusion materials: Instech USA). From 5 post-operative days onwards, the mice were fed a, high-fat liquid diet (L10049, Research Diets, USA; Supplementary Table 1) for 3, 6 or 12 weeks (HFLD-overfed, n=3 /group). The daily amount of feed administrated increased in time from 580 kCal/kg BW (t=0) to 750 kCal/kg BW (t= 3d), 870 kCal/kg BW (t= 7d), to the maximally tolerated amount of 920 kCal/kg BW (t= 19d; 27 kCal/day). After this, kCal/kg BW declined in time due to the maximized intake and increasing bodyweight (see Supplementary Figure 1). A control group of 10-weeks old male C57Bl/6J mice (Harlan) without catheter had *ad libitum* access to a solid high-fat diet (D06092602, Research Diets; Supplementary Table 1) for 3, 6 or 12 weeks (HFD-fed, n=3, 5 and 5 per group resp.)). As a reference for normal values, a third group (n=6) of male C57Bl/6J mice (Harlan NL) was included that received standard chow (Special Diets Services, #801730) and was sacrificed at the age of 17 weeks (comparable to 6 weeks fed HFD and HFLD). All mice had *ad libitum* access to water throughout the experiment. Mice were sacrificed around noon by decapitation after a brief sedation with CO_2/O_2 (70:30). Systemic blood was immediately collected into heparin-coated Pst LH microtainer tubes (BD, USA) and centrifuged. The resulting plasma was frozen in liquid N_2 and stored at -80°C. Epididymal fat pads were removed, weighed, frozen in liquid N_2 and stored at -80°C. The liver was removed, weighed, and parts were either fixed in 4% formaldehyde/PBS or frozen in liquid N_2 and stored at -80°C. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

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2.2 MRI analysis of whole-body adipose tissue

After 6 weeks of diet, whole body adipose volumes were assessed (n=3 mice/group) under ketamine-medetomidine anesthesia using a clinical 3.0T Philips Intera MRI scanner (Philips Healthcare, Best, the Netherlands). Coronal whole body images of mice in prone position were acquired with a receive-only surface coil (5 cm diameter) using a T1-weighted Turbo Spin Echo sequence (parameters: TE/TR=15/183 ms, NSA =3, slice thickness 1.3 mm, no slice gap, in plain resolution 1.5 x 1.5 mm, field of view 90 x 60 mm). The signal intensity was highest at the level of the used MRI coil and lower at the cranial and caudal ends of the mice. Therefore, the signal intensity of adipose tissue along the body axis was measured in each transversal section, a conversion factor was calculated and used to normalize each section to the mid-coil section. This intensity correction was implemented in MATLAB (The MathWorks, Natick, MA, USA) and enabled computerized separation of adipose tissue from other tissues in 3D. The processed image data sets were segmented and intra-abdominal (*i.e.* within the peritoneal cavity*)* and whole-body fat volumes were determined using Amira software (Template Graphic Software, Mercury Computer Systems). To segment images, a manual threshold was set to separate the high intensity signal of adipose tissue from the lower intensity signal of non-fatty tissues and background. A lower threshold was used to separate the complete animal from the background. This way the volumes of adipose tissue and of the complete animal were determined. This was followed by manual separation of visceral and non-visceral fat in the images to determine relative visceral fat volume.

2.3 Plasma analyses

Plasma levels of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma-glutamyl transpeptidase (γ-GT), alkaline phosphatase (AP) and bilirubin were determined in a Hitachi 911 analyzer (Roche/Hitachi, Indianapolis, IN). Plasma insulin was measured with the "Ultra Sensitive Mouse Insulin ELISA" kit (Chrystal Chem Inc, USA). Plasma TNFα was measured with the "Mouse TNFα ELISA MAX" kit (BioLegend, USA). Plasma IL-6 was measured with the "BD OptEIA Mouse IL-6 ELISA" kit (BD

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Biosciences,USA). For detection of FABP4 protein in plasma of 6 weeks HFD-fed and HFLD-overfed mice (all n=3/group), equal amounts of plasma were loaded on a polyacrylamidegel. Proteins were blotted on PVDF membrane and detected with a rabbit polyclonal FABP4 antibody (Cell Signaling, USA) and appropriate HRP-labeled 2nd antibody (DAKO). Chemiluminescent signals were detected using “Lumilight Plus” (Roche).

2.4 Oral glucose-tolerance test

Mice were fasted for 5 hours after which blood glucose (t=0) was measured in a drop of tail vein blood using an Elite Glucometer (Roche). In addition, a few drops of blood were collected in a heparin-coated PST LH Microtainer (BD, USA) to enable measurement of fasted insulin levels. Then mice orally received D-glucose (20% w/w solution; 1 g/kg BW). After 10, 20, 30, 60 and 90 minutes, blood glucose was measured in a drop of tail vein blood. The area under the curve was calculated by the trapezoidal method.

2.5 Biochemical liver analyses

Lipids were extracted from 50-100 mg of liver as described *[18]*. Triglycerides were measured using the "Trig/GB" kit (Roche). NEFA were measured using the "NEFA c" kit (WAKO, Germany). Cholesterol was measured using the "Cholesterol RTU" kit (Biomerieux, France). Phospholipids were measured using the "Phospholipids" kit (Spinreact, Spain).

2.6 Western blot analyses

50-100 mg of liver was homogenized in RIPA buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1% NP40, 5 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) with protease and phosphatase inhibitors (both Roche). Proteins (equal µg/lane) were separated by 10% SDS-PAAGE and transferred to PVDF membrane (Millipore). Membranes were blocked in 5% BSA/TBS-T (50 mM Tris, 150 mM NaCl, 0.1% Tween-20 (pH7.5), incubated with 1st antibody (P-Ser473-Akt, panAkt, P-Ser51-eIF2α or eIF2α (all Cell Signaling, USA)) and appropriate HRP-labeled 2nd antibody

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(all DAKO). Chemiluminescent signals were detected and quantified using “Lumilight Plus” (Roche) and a Lumi-Imager F1 (Roche).

2.7 (Immuno)histochemistry

Liver histology was assessed by staining formaldehyde-fixed liver sections with PicroSiriusRed (for fibrosis) and Periodic Acid Schiff (for glycogen) or by staining cryosections with Oil Red O (for steatosis). Macrophages (both infiltrating and liver-resident (Kupffer cells)) were detected in cryosections with an α-F4/80 antibody (AbD Serotec) and activated macrophages with an α-CD11b (Mac1) antibody (R&D systems). Infiltrating granulocytes and monocytes were stained with a Ly6/Gr1 antibody (BD Pharmingen).

2.8 Hepatic gene expression analyses

RNA was isolated from 50-100 mg frozen liver using Trizol reagent (Invitrogen, NL). cDNA was synthesized using locked oligo-dT primers (5’-TTT TTT TTT TTT TTVN-3’) and Superscript III reverse transcriptase (Invitrogen, NL). Quantitative PCR was carried out in a Lightcycler 2.0 with the "FastStart DNA Master^{Plus} SYBR Green I" kit (Roche). Primers are shown in supplementary Table 2. Raw Lightcycler data were analyzed using the LinReg program [19]. Measurements were corrected for between-session variation [20] and normalized for their 18S content.

2.9 Statistical analyses

Data are expressed as mean values $\pm$ SEM. All statistical tests were performed with SPSS 14.0. Data were first analyzed by two-way ANOVA to assess differences between the diet groups as a whole. Subsequently one-way ANOVA per time point and per diet was performed, followed by Student-Newman-Keuls post hoc comparisons. $P \leq 0.05$ was considered statistically significant.

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3. Results

3.1. Overfeeding a high-fat liquid diet results in a progressive increase in body, liver and fat-pad weights.

Feeding C57Bl/6J male mice *ad libitum* a solid, olive-oil based, high-fat diet (HFD) resulted in a continuous and modest bodyweight gain of 20% in a period of 12 weeks (Figure1A). This is comparable to the normal weight gain of chow-fed C57Bl/6J male mice of similar age. In contrast, overfeeding a high-fat liquid diet (HFLD) for the same period resulted in a ~2.5-fold increase in bodyweight (Figure 1A). The liver weights, both absolute and relative to bodyweight, were significantly increased in the HFLD-overfed mice only (Figure 1B and Supplementary Figure 2). The biggest effect was seen on the weight of the epididymal fat pads, especially after 6 and 12 weeks of HFLD feeding (Figure 1C and Supplementary Figure 2). Remarkably, the relative weights of the liver and fat pad returned to control values after 6-12 weeks of the HFD diet, whereas the effects persisted in HFLD-fed mice. Body fat volumes were also quantified using MRI imaging (Figure 1D). The body volume and total body fat volume of the mice correlated tightly with bodyweight and epidydimal fat pad weight, respectively (see Supplementary Figure 2). The percentage total body fat almost doubled in 6 weeks HFLD-overfed mice compared to 6 weeks HFD-fed mice (Figure 1E). Interestingly, the contribution of intra-abdominal fat to total body fat content was not different between HFD-fed and HFLD-overfed mice (Figure 1F).

3.2. In overfed mice liver steatosis is more persistent than in HFD-fed mice

A prominent accumulation of lipid droplets was seen in the liver from the earliest time point (3 weeks) onwards in both HFD-fed and HFLD-overfed mice, but the zonal distribution of the lipid droplets was remarkably different between both high-fat fed groups (Figure 2A and Supplementary Figure 3). Lipid accumulated predominantly in relatively large vesicles situated around the portal veins in the HFD-fed groups. In the HFLD-overfed mice, initially a homogeneous accumulation of small lipid vesicles was observed. However, after 12 weeks of overfeeding, also large lipid droplets were present around the portal veins in addition to

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the homogeneously distributed smaller lipid vesicles (Figure 2A and Supplementary Figure 3).

Liver triglyceride levels were >10-fold higher in HFD-fed and HFLD-overfed mice than in chow-fed mice (Figure 2B). Between 3 and 12 weeks on the high-fat diet, hepatic triglyceride content in the HFD-fed mice decreased, whereas it remained elevated in the HFLD-overfed mice (see Figure 2B). Hepatic cholesterol, free fatty acids and phospholipids were also significantly increased in both HFD-fed and HFLD-overfed mice compared to chow-fed mice (data not shown) The persistent steatosis in the HFLD-overfed mice was also reflected by the significantly increased hepatic mRNA expression of two markers of steatotic livers: peroxisome proliferator-activated receptor γ[21,22] (*Ppar*γ, Figure 2C) and fibroblast growth factor 21[23,24] (*Fgf21*; Figure 2C).

Remarkably, plasma TG levels decreased ~5-fold with time in mice on the HFD diet (Figure 2D). In mice on the HFLD diet, plasma TG levels were similar to those in chow-fed mice for the first 6 weeks on the diet, but decreased ~2-fold between 6 and 12 weeks on the diet. Plasma cholesterol levels were ~50% increased in both HFD-fed and HFLD-overfed mice from the earliest time point on the high-fat diet onwards (Figure 2D). Plasma NEFA levels were not significantly different at any time point between any of the diets (not shown). Plasma ALAT, ASAT, AP, γ-GT or bilirubin levels were not significantly elevated in the experimental groups (data not shown).

3.3. Overfeeding causes hyperinsulinemia, but no insulin resistance

Plasma glucose levels did not differ significantly between chow-fed, HFD-fed and HFLD-overfed mice in either the fasted or the non-fasted condition (not shown). In contrast, fasted insulin levels and insulin resistance, as defined by an Oral Glucose Tolerance Test (OGTT), were slightly but significantly increased in the *ad libitum* fed group compared to the chow- and overfed groups at 6 weeks of feeding (Figure 3A and Supplementary Figure 4A, respectively). However, non-fasted insulin levels were significantly higher in the 12 weeks overfed mice (Figure 4B). In the livers of 6 and 12 weeks HFLD-overfed mice, normal insulin

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signaling was still apparent: the degree of phosphorylation of Akt/PKB was increased (Figure 4C) and correlated positively with plasma insulin levels (R^2= 0.99; Supplementary Figure 4B). Phosphorylation of glycogen synthase kinase 3β (GSK3β), a target of Akt/PKB, was also observed in the livers of 6 and 12 weeks HFLD-overfed mice; this correlated with the phosphorylation of Akt/PKB (data not shown).

3.4. Increased lipogenesis and lipid uptake in livers of overfed mice

To uncover putative mechanisms involved in the observed steatosis, the expression of a number of key mediators of carbohydrate and lipid metabolism was studied. The mRNA expression of fatty acid synthase (*Fas*) and stearylCoA desaturase 1 (*Scd1*), both key factors in lipogenesis, were unchanged in HFD-fed mice but were increased in the HFLD-overfed mice (Figure 4A). In addition, the mRNA levels of fatty acid translocase (*Fat/CD36)* and fatty acid binding protein (*Fabp*), involved in fatty acid uptake and intracellular fatty acid binding respectively, were also exclusively increased in the livers of HFLD-overfed mice (Figure 4B). Moreover, plasma FABP4 protein levels differed substantially between HFD-fed and HFLD-overfed mice (Figure 4C).The mRNA levels of carnitine palmitoyltransferase 1 (*Cpt1a*), acetylCoA oxidase 1 (*Acox1*) and peroxisome proliferator-activated receptor α (*Ppar*α), all involved in fatty acid oxidation, were not different in time and between diets (data not shown). Similarly, the mRNA expression levels of phosphoenolpyruvate caboxykinase (*Pck*), glucokinase (*Gck*) and carbohydrate-responsive element-binding protein (*Chrebp*[25]), all involved in carbohydrate metabolism, were unchanged in all groups (data not shown).

3.5. Inflammation becomes apparent with time in HFLD-overfed mouse livers only.

Hepatic inflammation was assessed by immunohistochemical staining of liver sections and measuring hepatic mRNA expression of inflammation markers. Staining for the general macrophage marker F4/80 showed that the number of macrophages did not differ appreciably between chow-fed, HFD-fed and HFLD-overfed mice (Figure 5A). At the mRNA level, liver *F4/80* expression increased ~2-fold in HFLD-overfed mice compared to chow- and

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HFD-fed mice (Figure 5B). However, the number of CD11b/Mac1-positive (activated) macrophages was significantly higher in the HFLD-overfed mice at all times and increased with time to a ~10-fold difference at 12 weeks (Figures 5A and B). The mRNA expression of Toll-like receptor 4 (*Tlr4*), also expressed on macrophages, mirrored the *CD11b/Mac1* data (data not shown). Most remarkable was the induction of mRNA expression of myeloperoxidase (*Mpo*) from virtually absent in chow-fed and HFD-fed mice to ~1,500-fold higher levels in 12-weeks HFLD-overfed mice (Figure 5B). *Mpo* is expressed in neutrophils and monocytes, but also in Kupffer cells[26]. The more pronounced increase in *Mpo* than in *Mac1/CD11b* expression implies that the increase in *Mpo* expression in the livers of HFLD-overfed mice largely reflects infiltrating neutrophils. Indeed, numerous clusters of inflammatory cells containing Ly6/Gr1-positive neutrophils or monocytes were found in the livers of HFLD-overfed mice (Figure 5C).

None of the livers showed signs of extensive liver fibrosis except that of one 12-weeks HFLD-overfed mouse that showed extensive fibrosis (Supplementary Figure 5). This fibrosis co-localized with large inflammatory foci. In agreement, the hepatic mRNA expression of α1procollagen(I) was not different between the different experimental groups (data not shown).

Hepatic oxidative stress and lipid peroxidation are implicated in the progression of fatty livers to NASH[27-29]. However, the fraction of oxidized glutathione (GSH/GSSG) did not differ between the experimental groups (Supplementary Figure 6A). Furthermore, the expression of oxidative stress markers Nrf2, catalase and *Cyp2E1* was not different between HFD-fed and HFLD-overfed mice (Supplementary Figure 6B).,. The expression of heme oxygenase 1 (*Ho-1*), which may respond to oxidative stress[27,30], was ~3-fold induced in the livers of HFLD-overfed mice (Figure 6A).

Fatty-acid accumulation and exposure to cytokines both induce endoplasmatic reticulum (ER) stress, which, in turn, causes increased expression of the transcription factor C/EBP-homologous protein (*Chop*)[31]. Significantly increased levels of *Chop* mRNA were found exclusively in the livers of 12 weeks HFLD-overfed mice (Figure 6B). In accordance,

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increased phosphorylation of elongation initiation factor 2α (eIF2α), the first signaling intermediate of the stress kinases[31], was found in this group only (Figure 6C). In contrast, no differences in the splicing of *Xbp1* mRNA were observed between the respective groups, which excludes a role for the stress kinase Ire1α in the observed ER stress (data not shown).

With the exception of one (12-weeks HFD-fed) mouse, all chow and HFD-fed mice displayed a normal glycogen content (Figure 7). In contrast, all 12-weeks HFLD-overfed mice had virtually no glycogen in their hepatocytes. The HFLD mice gradually lost their liver glycogen, because only ~one-third of the 6-weeks HFLD group had decreased amounts of glycogen in their hepatocytes. The loss of glycogen correlated with the increased expression of inflammation markers.

3.6 Overfeeding leads to inflamed adipose tissue with compromised metabolic function

Plasma tumor necrosis factor α (TNFα) levels were significantly increased at all time points in the HFLD-overfed mice only (Figure 8A). Plasma interleukin-6 (IL6) levels also tended to be increased, though this was not statistically significant (Supplementary Figure 7). mRNA analyses showed an approximately 20 times higher expression of both *Tnfα* and *Il6* in adipose tissue compared to liver (Figure 8B; only WAT shown); hence the majority of the increased plasma IL-6 and TNFα levels in the overfed mice originates from adipose tissue. In addition, the mRNA expression of the general macrophage marker *F4/80* and activated macrophage marker *CD11b/Mac1* was also significantly increased in WAT of HFLD-overfed mice (Figure 8C). In contrast, the mRNA expression of adiponectin, an anti-inflammatory adipokine, was significantly reduced in WAT of HFLD-overfed mice (Figure 8D). Furthermore, the expression of vascular endothelial growth factor α (*Vegfα;* Figure 8E*),* involved in vascularization and often linked to adipogenesis[32], and the expression of adipocyte differentiation markers *Pparγ* and *c/Ebpα*, were significantly decreased in WAT of HFLD-overfed compared to HFD-fed mice (Figure 8F; only *c/Ebpα* shown). Similar results were observed for the expression of the fatty-acid transporter *CD36* and the lipid chaperone *Fabp4* (*aP2*) (Figure 8F; only *aP2* shown).

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Discussion

Preferably, an animal model of NAFLD should display the same liver pathology as seen in human NAFLD, *i.e.* steatosis, steatohepatitis and steatohepatitis plus fibrosis. In addition, this pathology should occur within the metabolic setting that is often present in human NAFLD: obesity, insulin resistance, dyslipidemia, and altered serum adipokine levels. This study used intragastric overfeeding of mice with a high-fat liquid diet *[17]* to induce obesity and tested whether NAFLD as well as the associated metabolic abnormalities could be recreated in time. This proved to be so and as such, our data are a longitudinal extension of the data from Deng *et al [17]* with a comparable but slightly less severe phenotype at the late stage of overfeeding. Although the mice in both studies are equally obese, a remarkable difference between this study and that of Deng *et al [17]* is the respective absence and presence of insulin resistance. The latter may be explained by the use of different diet compositions in both studies. The lipid composition is comparable between the diets in both studies, both in terms of caloric value as well as percentage of saturated lipids. However, whereas the Deng diet uses solely (high glycemic index) glucose as carbohydrate source, (low glycemic index) maltodextrin is the main carbohydrate source in the present study. A low glycemic index diet has been shown to reduce diabetes incidence and improve diabetes control[33]. The sometimes relatively high variation in the markers we studied can be readily explained by the experience from human studies that only a fraction of patients with steatosis progress to steatohepatitis and fibrosis, and that not all patients do so at the same moment. Our rare observation of extensive fibrosis in mice corresponds with this relatively infrequent sequel of steatosis.

The livers of the overfed mice were metabolically characterized by an increased expression of (pericentrally located) lipogenic genes (*Fas, Scd1* and *Pparγ*), combined with a high portal inflow of dietary FFAs. These nutritional and metabolic sources of fat explain the observed initial homogeneous lipid accumulation in the livers of these mice. At later stages, the basal expression of lipolytic genes (*Cpt1a, Acox1* and *Pparα*), combined with the maximal inflow of dietary FFAs, cause the observed periportal lipid accumulation on top of

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the initially homogeneous steatosis. Unfortunately, the zonation of hepatic lipid accumulation has not been determined in other diet-induced obesity studies [17,34-37]. Carbohydrate metabolism seems to play at best a minor role in our overfed mouse model, since the expression of key factors in gluconeogenesis and glycolysis is not altered.

The mRNA expression of peroxisome proliferator-activated receptor γ (*Pparγ*) and fibroblast growth factor 21 (*Fgf21*) is significantly induced only in the livers of the HFLD-overfed mice. Previous studies have shown overexpression of both *Pparγ* and *Fgf21* in the steatotic levers of obese, diabetic mice[38,39] and in liver biopsies from NAFLD patients[23,24,40]. In addition, it has been shown that PPARγ may induce FGF21 expression in mature murine 3T3L1 adipocytes[41]. Both PPARγ and FGF21 have profound effects on (lipid) metabolism, but a causal relationship between PPARγ and FGF21 in liver steatosis or steatohepatitis has not yet been established. Our data suggest such a relationship, but this requires further investigation. PPARα has also been reported to induce liver *Fgf21*, but since we find no increased expression of *Pparα* in liver of HFLD-overfed mice, PPARα is a less likely candidate for *Fgf21* induction

Hepatic steatosis *per se* is insufficient to initiate steatohepatitis, as mice *ad libitum* fed a solid high-fat diet did develop steatosis to a similar extent as mice overfed a near-identical high-fat liquid diet, but showed no increased prevalence of NASH (see also [1,36,42]. According to the “two hit” hypothesis[4,43], oxidative stress and pro-inflammatory cytokine-mediated hepatocyte injury are two putative mechanisms thought to be involved in progression to NASH. In our overfed mouse model we find no increased expression of oxidative stress markers, but we do observe increased cytokine levels.

We hypothesize that the origin for progression to NASH may very well lie within the inability of the overfed mice to adapt to the caloric overload, which leads to the severely increased adipose mass that is present in these mice. Whereas the *ad libitum* fed mice decrease their caloric intake which even results in a reduced adipose mass after 6 and 12 weeks of HFD feeding, the adipose mass in the overfed mice more than doubles from 3 to 12 weeks of overfeeding. Several studies show a relationship between adipose tisue

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expandibility and susceptibility to the metabolic syndrome [44-47]. Two non-exclusive models have been proposed to account for this phenomenon: In the first, excessive fat accumulation is associated with chronic inflammation, increased cytokine production and altered adipokine secretion of WAT. Both cytokines and adipokines influence metabolism in peripheral tissues. In the second, lipotoxicity, model, adipocytes are metabolically changed which results in a decreased lipid storage capacity and increased lipid outflow. This causes lipotoxicity in peripheral organs. Our overfed mouse model likely displays both mechanisms since increased expression of inflammation markers and decreased expression of adiponectin, adipocyte differentiation markers *Pparγ* and *c/Ebpα* and the lipogenic genes *Fat/CD36* and *Fabp4/aP2* are observed in WAT of HFLD-overfed mice compared to WAT of HFD-fed mice. In addition, increased plasma FABP4 and increased hepatic expression of *Fat/CD36* and *Fabp* are observed in HFLD-overfed mice compared to HFD-fed mice. These observations suggest an increased flux of fatty acids through the overfed mouse livers, similar to that observed in NAFLD patients[12,13], making the lipotoxity hypothesis a likely explanation for the observed steatohepatitis in our overfed mice.

Increased visceral fat mass along with a changed serum adipokine and cytokine profile is prevalent in human NAFLD (Fan and Farrell[48] and references therein). Blood coming from visceral fat drains directly into the liver. It is therefore tempting to speculate that the increased inflow of pro-inflammatory cytokines and fatty acids and decreased inflow of anti-inflammatory adiponectin leads to the observed increase of liver macrophages and other infiltrating inflammatory cells. The above mechanisms may also account for other observed phenomena in our overfed mouse model: increased phosphorylation of eIF2α and mRNA expression of *Chop*, both markers of endoplasmatic reticulum (ER) stress, and decreased liver glycogen storage. Previous studies have shown that fatty acids and cytokines are associated with ER stress, both in rodents[49,50] and in NAFLD patients[51]. In addition, fatty acids may decrease glucose conversion into glycogen [52,53].

In conclusion, the overfed mouse model displays the characteristics of human NAFLD within the appropriate metabolic setting, *i.e* obesity through overcaloric intake of a high-fat

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diet. It is shown that the transition from steatosis to NASH coïncides with major changes in adipose tissue. The causal relationship between NASH and the compromised function of inflamed adipose tissue requires further study.

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Abbreviations

HFD : High-fat diet

HFLD: High-fat liquid diet

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Figure Legends

Figure 1. Changes in bodyweight (**A**), liver weight (**B**) and fat-pad weight (**C**) in C57Bl6/J mice *ad libitum* fed a solid high-fat diet (HFD) or overfed a high-fat liquid diet (HFLD) for 3, 6 or 12 weeks (HFD: n=3, 5 and 5 per group, resp; HFLD: n=3 per group, resp). Chow-fed mice (n=6) were included as reference for normal values. **(D)** MRI quantification of total body fat in C57Bl6/J mice *ad libitum* fed a solid high-fat diet (HFD) or overfed a high-fat liquid diet (HFLD) for 6 weeks (n=3/group). **(E)** Volume % total body fat as determined by MRI. **(F)** ratio of intra-abdominal fat volume to total body fat volume as determined by MRI. Values represent means± SEM. *: significantly different (P<0.05); #: significantly different from other groups within diet (p<0.05); γ: significantly different from chow (p<0.05).

Figure 2. **(A**) Representative Oil Red O staining of liver sections of C57Bl6/J mice chow-fed (chow, n=6/group), *ad libitum* fed a solid high-fat diet (HFD: n=3, 5 and 5 per group, resp) or overfed a high-fat liquid diet (HFLD: n=3/group, resp) for 3, 6 or 12 weeks (magnification: 5x). **(B)** Liver triglycerides (TG) of these mice. **(C)** mRNA expression of *Ppar*γ and *Fgf21* relative to 18S RNA in the livers of these mice. **(D)** Plasma triglycerides and cholesterol of these mice. Values represent means ± SEM. *: significantly different (p<0.05); #: significantly different from other groups within diet (p<0.05); γ: significantly different from chow (p<0.05); $: significantly different from all other groups (p<0.05).

Figure 3. **(A)** Plasma insulin levels of C57Bl6/J mice *ad libitum* fed a solid high-fat diet (HFD: n=3, 5 and 5 per group, resp) or overfed a high-fat liquid diet (HFLD: n=3/group, resp.) for 3, 6 or 12 weeks. Values represent means± SEM. $: significantly different from all other groups (p<0.05). **(B)** Amount of phospho-Akt/PKB (P-AKT) and total Akt on a Western blot of total protein isolated from livers of C57Bl6/J mice *ad libitum* fed a solid high-fat diet (HFD: n=5/ group, resp) or overfed a high-fat liquid diet (HFLD: n=3/group, resp.) for 6 or 12 weeks. Two

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representative samples of each group were loaded on the gel. Equal amounts of protein were loaded in each lane.

Figure 4. mRNA expression of *Fas* and *Scd1* **(A)** and of *Fabp* and *Fat*/*Cd36* **(B)**, relative to 18S RNA in the livers of chow-fed (n=6), HFD-fed (n=3, 5 and 5 per group, resp) and HFLD-overfed mice (n=3/group, resp). Values represent means± SEM. *: significantly different (p<0.05). **C.** Detection of FABP4 protein in plasma of 6 weeks HFD-fed and HFLD-overfed mice (all n=3/group). Equal amounts of plasma were loaded in each lane.

Figure 5. **(A**) Liver sections of C57Bl6/J mice chow-fed ((n=6), *ad libitum* fed a solid high-fat diet (HFD: n=5/group) or overfed a high-fat liquid diet (HFLD: n=3/group) for 6 or 12 weeks stained for the general macrophage marker F4/80 (top row) or for Mac1/CD11b (activated macrophages). Magnification: 10x. **(B)** mRNA expression of *F4/80*, *Mac1/CD11b* and myeloperoxidase (*Mpo*) relative to 18S RNA in the livers of these mice. Values represent means± SEM *: significantly different (p<0.05). **(C)** Liver sections of these mice stained for Ly6/Gr1 (granulocytes, monocytes). Magnification: 20x.

Figure 6. mRNA expression of *Ho-1* **(A**) and *Chop* **(B)**, relative to 18S RNA in the livers of chow-fed (n=6), HFD-fed (n=3, 5 and 5 per group, resp.) and HFLD-overfed (n=3/group, resp.) mice. Values represent means± SEM. *: significantly different (p<0.05); γ: significantly different from chow (p<0.05); **(C)** Amount of phospho-eIF2α (P-eIF2α; lower panel) and total eIF2α (top panel) on a Western blot of total protein isolated from livers of C57Bl6/J mice chow-fed (C), *ad libitum* fed a solid high-fat diet (HFD) or overfed a high-fat liquid diet (HFLD) for 6 or 12 weeks (n=3-5 per group). Two representative samples of each group were loaded on the gel. Equal amounts of protein were loaded in each lane.

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Figure 7. Liver sections of C57Bl6/J mice chow-fed (n=6), *ad libitum* fed a solid high-fat diet (HFD: n=5/group, resp) or overfed a high-fat liquid diet (HFLD: n=3/group, resp.) for 6 or 12 weeks stained for glycogen content by Periodic acid Schiff staining. Magnification: 5x. A representative section for each group is shown.

Figure 8. **(A)** TNFα in plasma of C57Bl6/J mice chow-fed (chow), *ad libitum* fed a solid high-fat diet (HFD) or overfed a high-fat liquid diet (HFLD) for 6 or 12 weeks (all n=3/group). mRNA expression of *Tnfα* **(B)** *F4/80* (**C** left panel) and *Mac1/CD11b* (**C** right panel), adiponectin **(D),** *Vegfα* **(E)** and *c/EBPα* (**F** left panel) and *aP2* (**F** right panel) relative to 18S RNA in WAT of these mice (all n=3/group). Values represent means± SEM. *: significantly different ($p<0.05$); γ: significantly different from chow ($p<0.05$).

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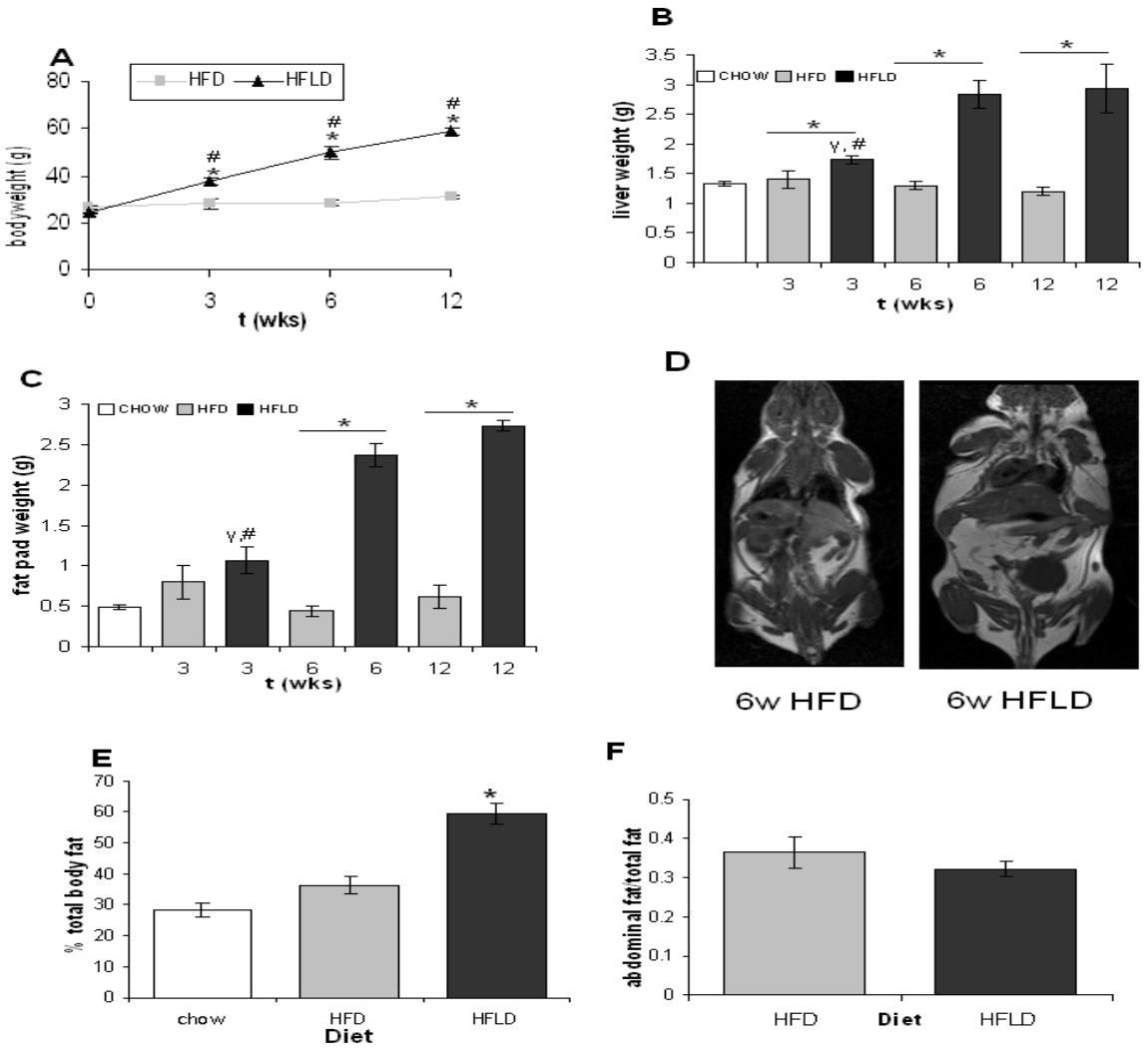

Figure1

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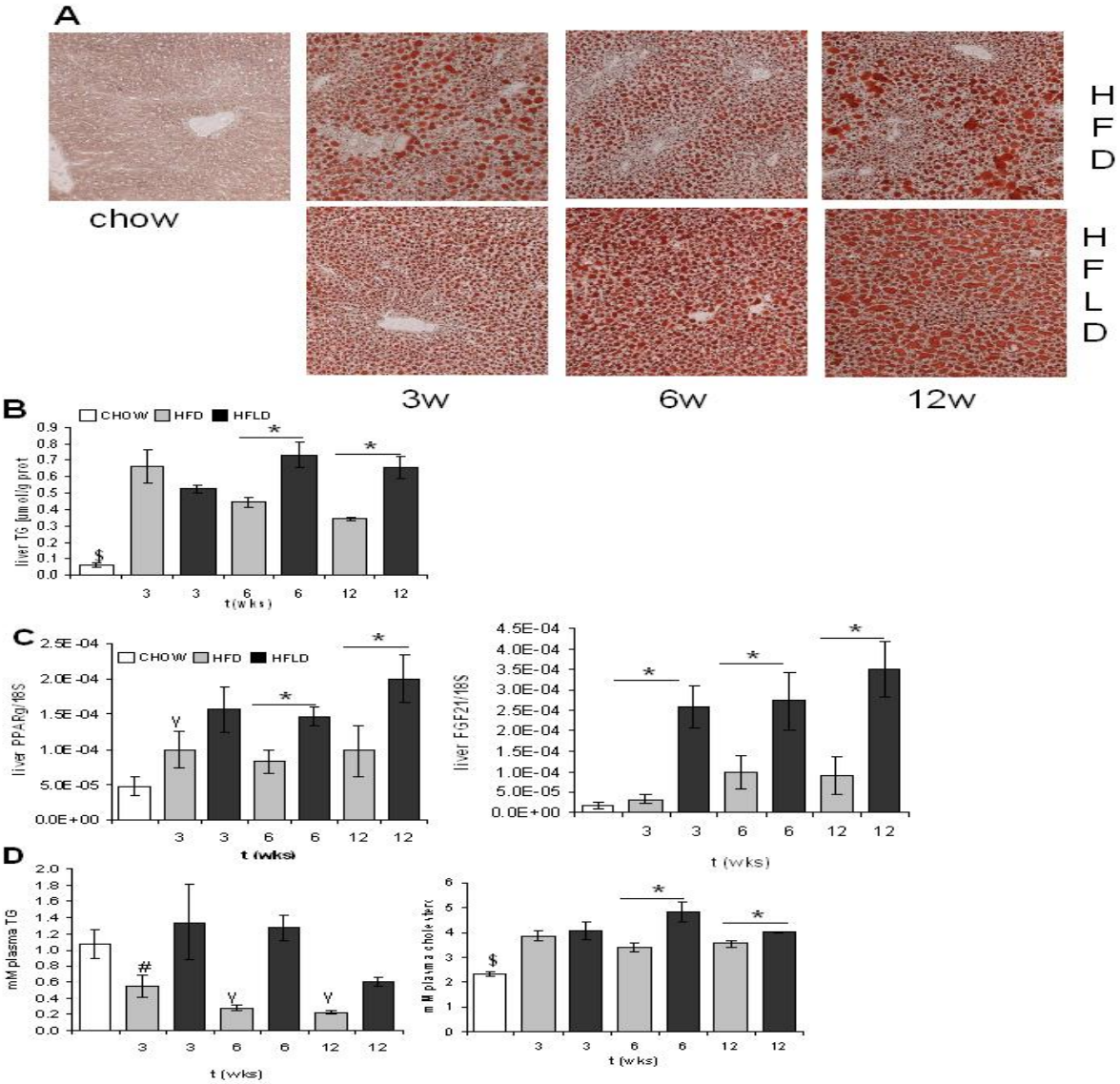

FIGURE 2

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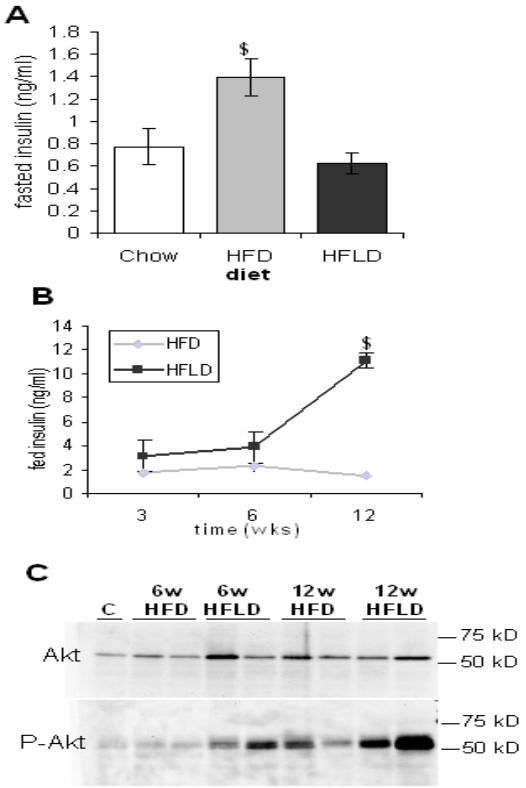

FIGURE 3

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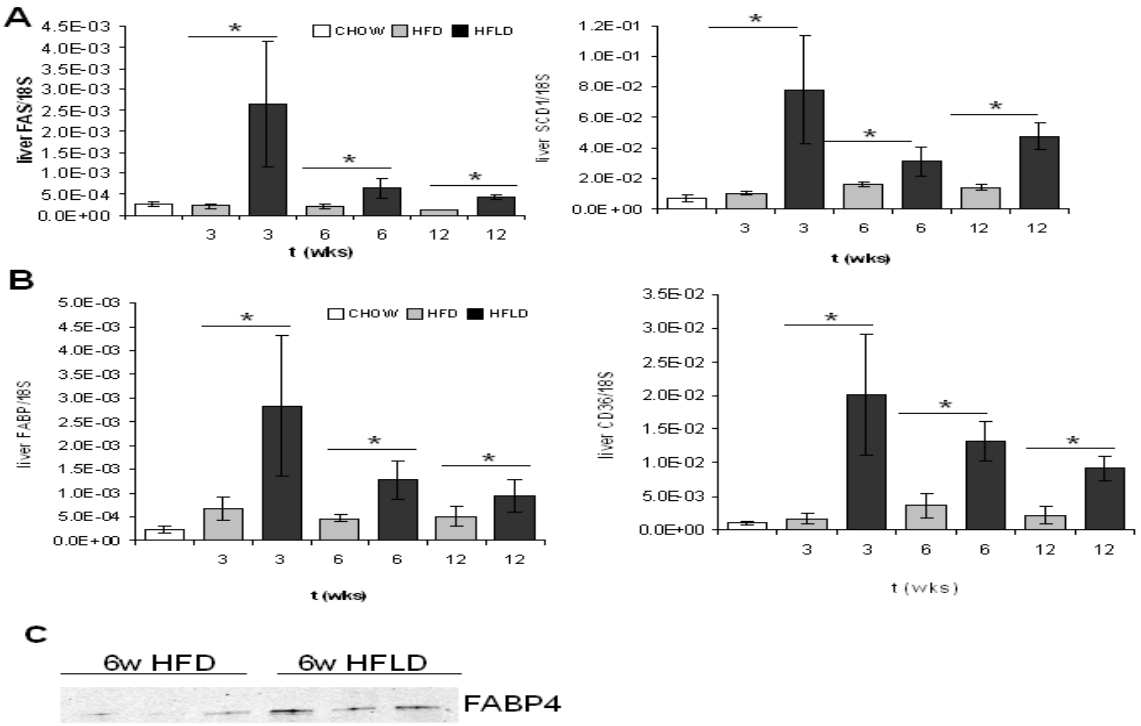

FIGURE 4

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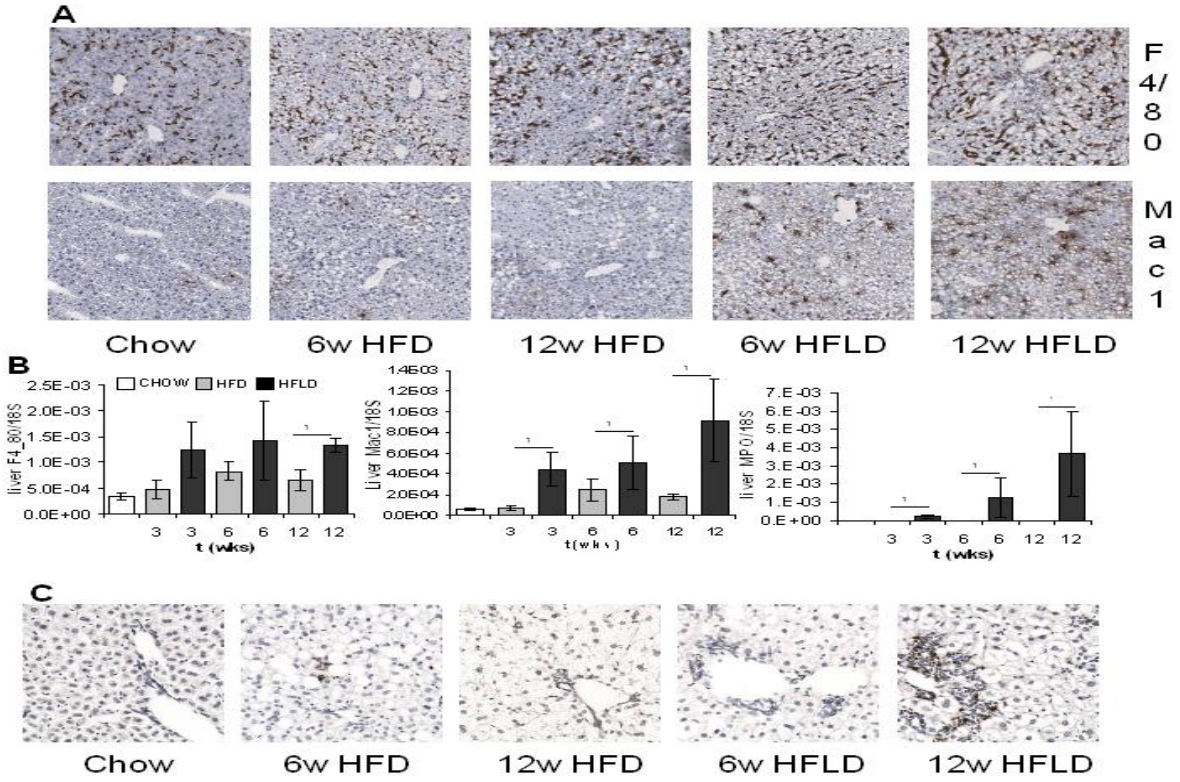

FIGURE 5

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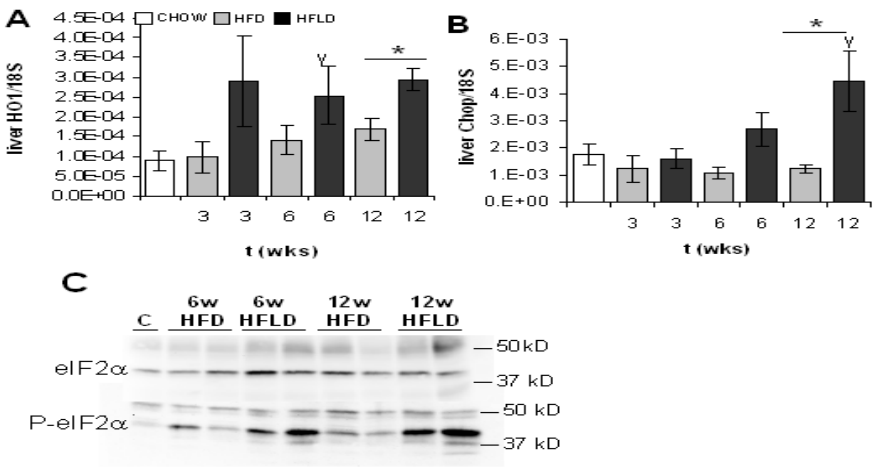

FIGURE 6

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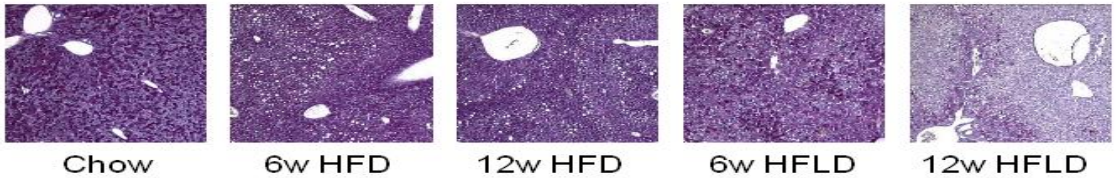

FIGURE 7

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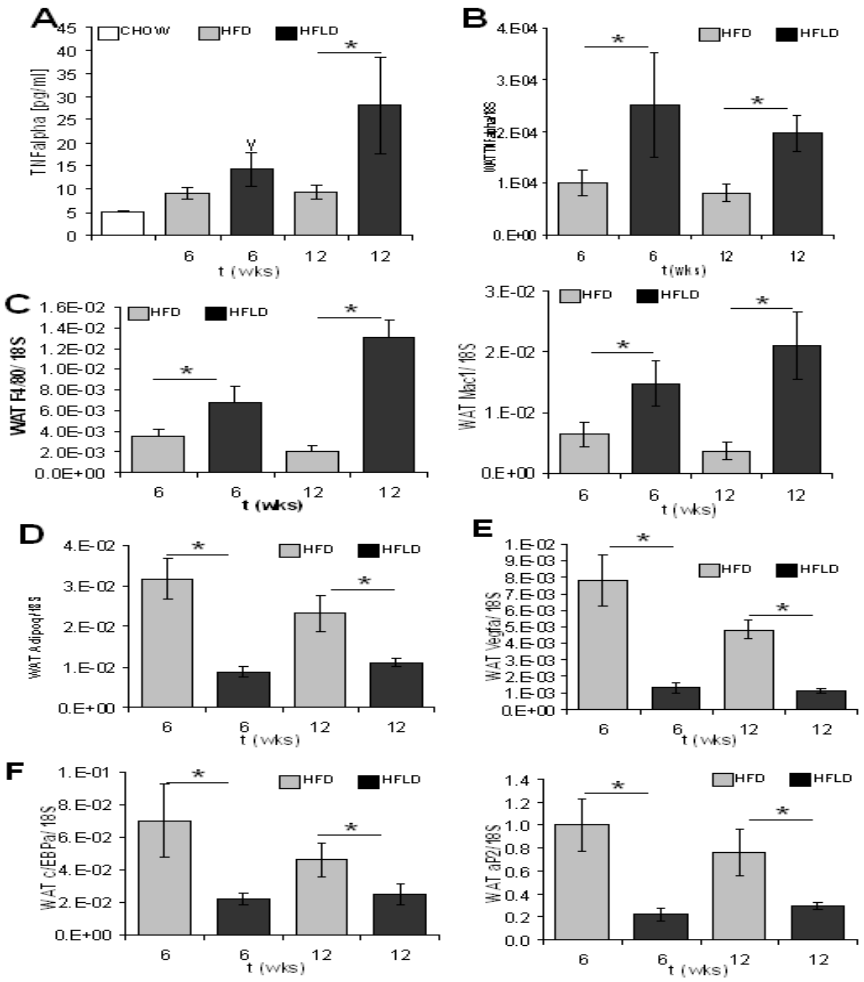

FIGURE 8

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Research highlights

The consecutive stages of NAFLD can be recreated in time in an overfed mouse model

Pparγ and *Fgf21* are induced in the livers of HFLD-overfed mice only

Steatohepatitis coïncides with obesity, hyperinsulinemia and loss of liver glycogen

Steatohepatitis coïncides with the occurrence of hepatic endoplasmatic reticulum stress

Adipose tissue of overfed mice is inflamed and has reduced adiponectin expression

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