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# Early diet-induced non-alcoholic steatohepatitis in APOE2 knock-in mice and its prevention by fibrates

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***Background/Aims*: The molecular mechanisms leading to Non-Alcoholic Steatohepatitis (NASH) are not fully understood. In mice, NASH can be inhibited by fenofibrate, a synthetic agonist for the nuclear receptor peroxisome proliferator activated receptor alpha, which regulates hepatic triglyceride metabolism. This study aimed to elucidate the relation between steatosis and inflammation in NASH in a human-like hyperlipidemic mouse model.**

***Methods*: Liver phenotype and gene expression were assessed in APOE2 knock-in mice that were fed a western-type high fat diet with or without co-administration of fenofibrate.**

***Results*: In response to a western diet, APOE2 knock-in mice developed NASH characterized by steatosis and inflammation. Strikingly, macrophage accumulation in the liver preceded the steatosis during progression of the disease. This phenotype was in line with gene expression patterns, which showed regulation of two major groups of genes, i.e. inflammatory and lipid genes. Fenofibrate treatment decreased hepatic macrophage accumulation and abolished steatosis. Moreover, a marked reduction in the expression of inflammatory genes occurred immediately after fenofibrate treatment.**

***Conclusions*: These data indicate that inflammation might play an instrumental role during the development of NASH in this mouse model. Inhibition of NASH by fenofibrate may be due, at least in part, to its inhibitory effect on pro-inflammatory genes.**



***Keywords*: Cholesterol; Hyperlipidemia; Microarray; Non-alcoholic steatohepatitis; PPARa; Triglyceride; APOE; Steatosis; Inflammation; Western diet**

## 1. Introduction

Non-alcoholic steatohepatitis (NASH) is characterized by pathological alterations ranging from steatosis and inflammation to cell degeneration, fibrosis and cirrhosis [1]. The pathogenesis of NASH remains poorly understood. It is a component of the metabolic syndrome and therefore frequently associated with hyperlipidemia [2]. Indeed, several mouse models of hyperlipidemia, such as ldlr$^{-/-}$

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and apoe$^{-/-}$ mice develop steatohepatitis upon high-fat feeding [18,19].

In order to study the development of NASH and the relationship of steatosis and inflammation, we used the 'humanized' APOE2 knock-in (APOE2KI) mouse, in which the human APOE2 allele replaces the murine apoe gene. These mice express human APOE2 under the control of the endogenous promoter sequences in a tissue specific manner and at physiological levels. APOE2 has a markedly reduced affinity for the LDL receptor, leading to a plasma lipoprotein profile resembling human type III hyperlipoproteinemia (HLP) [3].

Similar to humans, APOE2KI mice are responsive to lipid-lowering drugs such as fibrates, ligands for PPARα [3]. This nuclear receptor enhances the expression of genes involved in fatty acid (FA) uptake, esterification and β-oxidation [4] and has been shown to reverse steatohepatitis in mice [21]. Accordingly, fibrate was administrated to investigate the molecular mechanisms leading to the inhibition of NASH.

In this study, APOE2KI mice were fed a high-fat diet in the absence or presence of fenofibrate. The changes in plasma lipids, liver phenotype and gene expression were followed in time to discriminate between early (primary) and late (secondary) events related to NASH.

## 2. Materials and methods

### 2.1. Mice and diet

APOE2KI mice [3] were housed under standard conditions given free access to food and water. Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University.

Ninety homozygote female mice, 13 week old, were divided into groups of 10. One group was kept on standard chow. Four groups were fed a western diet, containing 17% casein, 0.3% DL-methionine, 34% sucrose, 14.5% cornstarch, 0.2% cholesterol, 5% cellulose, 7% CM 205B, 1% vit 200, 21% butter (diet 1635, Scientific Animal Food and Engineering, Villemoisson-sur-orge, France) for 2, 4, 7 and 21 days. Four groups were put on the western-diet completed with 0.2% Fenofibrate (F6020, Sigma Aldrich, Zwijndrecht, the Netherlands) for 2, 4, 7 and 21 days. Blood samples were taken from the tail after a 4-h fast and collected in glass capillaries, coated with heparin and diethyl *p*-nitrophenyl phosphate [5] (D9286, Sigma Aldrich, Zwijndrecht, The Netherlands). Mice were sacrificed by cervical dislocation. Tissues were isolated and snap-frozen in liquid nitrogen and stored at −80 °C or fixed in 4% formaldehyde/PBS.

### 2.2. Plasma parameters

Total plasma cholesterol and triglyceride (TG) were measured (1489232, Cholesterol CHOD-PAP, Roche, Almere, The Netherlands; 337-B, TG GPO-trinder, Sigma Aldrich, Zwijndrecht, The Netherlands) according to manufacturer's protocols on a Benchmark 550 Micro-plate Reader (170-6750XTU, Bio-Rad, Veenendaal, The Netherlands).

Lipoprotein profiles were determined on pooled plasma samples from 10 mice using an AKTA Basic chromotography system with a Superose 6PC 3.2/30 column (Amersham Biosciences, Roosendaal, The Netherlands).

### 2.3. RNA isolation

Total RNA was isolated from frozen tissues homogenized in TriReagent (T9424, Sigma Aldrich, Zwijndrecht, The Netherlands) with the MiniBeadBeater (3110BXEUR, Biospec Products, Bartlesville, USA). RNA clean-up was performed using Qiagen RNeasy Mini Kit (74104, Qiagen, Venlo, The Netherlands). Quality and quantity were determined with Agilent2100 Bioanalyzer and RNA 6000 NanoLabChip (5065-4476, AgilentTechnologies, Amstelveen, the Netherlands). Applications were done according to manufacturer's protocols.

### 2.4. cDNA synthesis and microarray hybridization

Pools of 10 mice were made from equal amounts of RNA from each mouse. Six micrograms of pooled liver RNA was used in cDNA synthesis. Samples were hybridized to Affymetrix Mouse Expression Array 430A (900412, Affymetrix UK Ltd, High Wycombe, UK) according to manufacturer's instructions.

### 2.5. Microarray analysis

Raw data from the microarrays was analyzed using GeneChip Microarray Suite 5.0 (MAS 5.0, Affymetrix) and Data Mining Tools 3.1 (DMT 3.1, Affymetrix). To identify regulated genes, ANOVA analysis was applied based on probe level information with, R (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org) was used. After normalization, a model was developed, based on probe level intensity for each probe set.

Genes were selected according to the *P*-value of the Diet/Treatment effect for each delay coefficient (four *P*-values per probe set) and for the multi-testing problem Bonferroni correction with a threshold of $1\times10^{-6}$ ($\sim0.05/(22{,}000\times3)$ (number of probe set×number of test by probe set) was used. Genes with a *P*-value lower than $10^{-6}$ were selected.

### 2.6. Immunohistochemistry

Four micrometers paraffin embedded liver sections were stained with Heamatoxillin/Eosin (HE). Seven micrometers frozen-cut liver sections were fixated in acetone and stained with CD68 (FA11), Mac1 (M1/70) and Nimp1 antibodies (granulocytes) as described before [6].

### 2.7. Hepatic lipid analysis

Approximately, 50 mg of frozen liver tissue was homogenized for 30 s at 5000 rpm in a closed tube with 5.0 mm glass beads and 1.0 ml SET buffer (Sucrose 250 mM, EDTA 2 mM and Tris 10 mM) [7]. Complete cell destruction was done by two freeze–thaw cycles and three times passing through a 27-gauge syringe needle and a final freeze–thaw cycle. Protein content was measured with the BCA method (23225, Pierce, Rockford, IL, USA). TG were measured as described above. Protocols were done according to manufacturers instructions.

### 2.8. Real-time quantitative PCR

Prior to real-time quantitative PCR (QPCR), liver RNA pools of each group were reverse transcribed with the iScript cDNA synthesis kit (170-8891, Bio-Rad, Veenendaal, The Netherlands) according to manufacturers instructions. cDNA quantification was done by QPCR on an ABI Prism 7700 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) with Mastermix Plus kit for Sybr Green I (RT-SN2X-03+*, Eurogentec, Seraing, Belgium). For each gene, a standard curve was generated. Normalization was done with cyclophillin A. Specific primers sets (Table 2) were developed with Primer Express 1.5 (Applied Biosystems). Data were analyzed with SDS 1.9.1 (Applied Biosystems).