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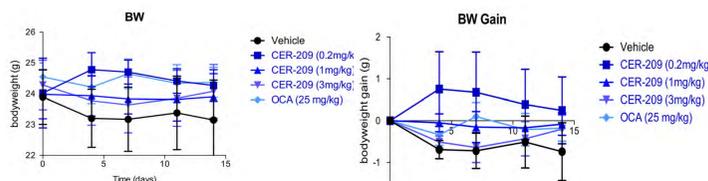
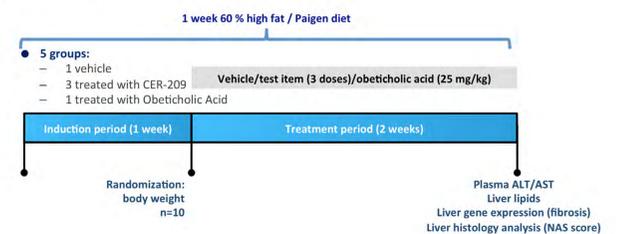
Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are increasing as a consequence of growing worldwide obesity/diabetes. NAFLD is the most prevalent chronic liver disease, affecting 20–40% of the general population, and approximately one-third of patients with NAFLD will progress to NASH. While no specific treatments are yet available, recent AASLD guidelines recommend weight loss, life-style changes to incorporate more physical activity, control of hyperglycemia and treatment of hyperlipidemia with statins to lower lipids through regulation of low-density lipoprotein (LDL) cholesterol. Reverse lipid transport (RLT), driven by high-density lipoprotein (HDL) metabolism, controls the transfer of cholesterol from non-hepatic cells to the liver, where it is excreted from the body in the form of bile acids and unesterified cholesterol. The capacity of HDL particles to mobilise cholesterol from atherosclerotic plaque confers on them a protective effect against heart disease (see Poster n° : P-914). HDL-directed lipid elimination by the liver could impact fatty liver and steatohepatitis; however, this has never been studied in NAFLD and NASH. Here we used a new in vivo mouse model of NAFLD/NASH induced using high fat/cholesterol/cholesterol diet over a short-term period to determine whether CER-209, a hepatic G protein-coupled receptor belonging

to a new class of P2Y13 receptor (P2Y13r) agonists and designed to improve HDL metabolism (i.e. HDL elimination), could also have a marked effect on liver metabolism. We have recently demonstrated in vivo CER-209 an agonist of P2Y13 receptor is key partner in the HDL metabolism and reverse cholesterol transport process, and thereby promoting atherosclerosis protection in mice. The data support a mechanism where the stimulation of the HDL uptake or endocytosis by the liver via P2Y13r pathway activation promotes cholesterol catabolism by the liver, secretion in the gallbladder and final fecal elimination.

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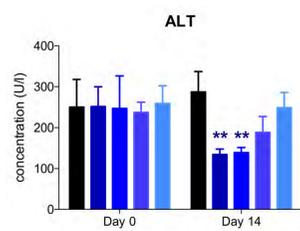
### Design of the Pilot study

◆ C57BL/6J mice (male, 8 week old)



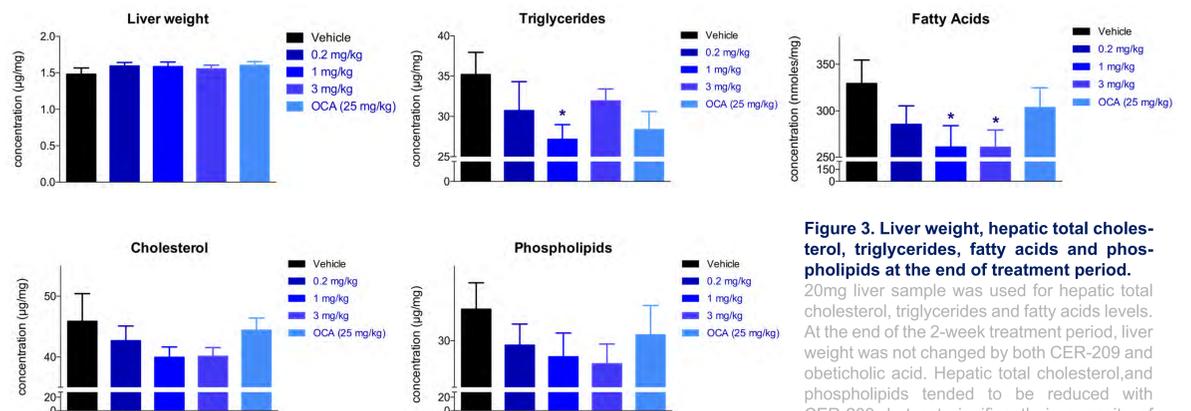
**Figure 1. Body weight and body weight gain during the 2-week treatment period.**

60% high fat/1.25% cholesterol/0.5% cholic acid diet with a 20% 2-hydroxypropyl beta-cyclodextrin QD oral gavage over 1 week induced a 1.8g reduction in body weight. At randomization (1 week of diet), mean body weight was 24g.



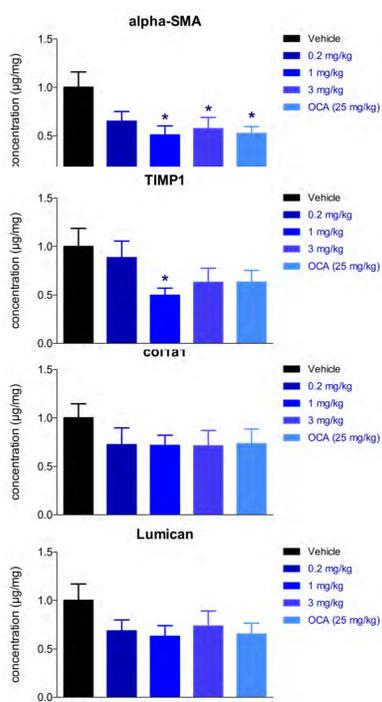
**Figure 2. Plasma ALT and AST levels at randomization and at the end of treatment period.**

At randomization (1 week of diet), mean plasma ALT and AST levels were 249 and 286 U/L, respectively. \*p<0.05 and \*\*p<0.01 vs. vehicle.



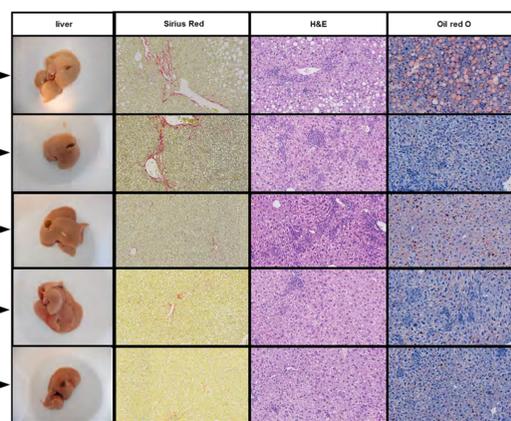
**Figure 3. Liver weight, hepatic total cholesterol, triglycerides, fatty acids and phospholipids at the end of treatment period.**

20mg liver sample was used for hepatic total cholesterol, triglycerides and fatty acids levels. At the end of the 2-week treatment period, liver weight was not changed by both CER-209 and obeticholic acid. Hepatic total cholesterol, and phospholipids tended to be reduced with CER-209, but not significantly in opposite of Triglycerides and FFA which were significantly reduced at 1 mg/kg and 3 mg/kg doses respectively.



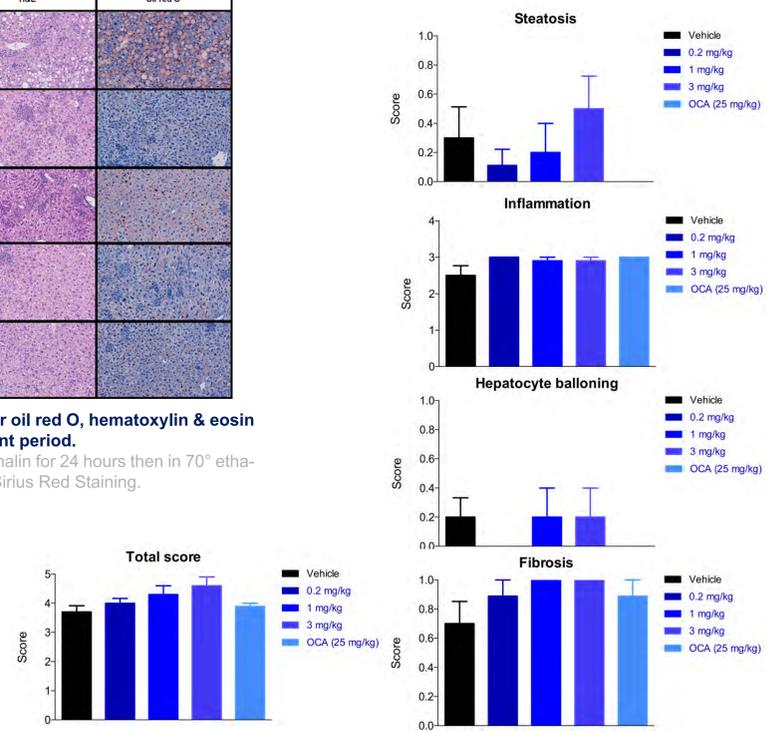
**Figure 4. Hepatic gene expression for alpha-SMA, collagen 1 alpha 1, TIMP1 and lumican at the end of treatment period.**

Hepatic gene expression (figure 6) showed a significant reduction of alpha-smooth muscle actin gene expression with CER-209 at 1 and 3 mg/kg and with obeticholic acid. Both CER-209 and obeticholic acid also showed a reduction in Collagen 1 alpha 1, TIMP1 and lumican gene expression, but not significantly. alpha-Smooth Muscle Actin (a-SMA): its presence in perisinusoidal cells is characteristic of liver fibrosis. collagen 1 alpha 1 (coll1a1): changes may parallel tissue collagen content/liver fibrosis development tissue inhibitor metalloproteinase 1 (TIMP1): has been shown to promote liver fibrosis in animals and humans. Lumican: a collagen-associated protein, small leucine-rich proteoglycan, known to play a role in collagen fibrillogenesis and the development of hepatic fibrosis



**Figure 5. Representative staining for oil red O, hematoxylin & eosin and Sirius red at the end of treatment period.**

0.5cm<sup>3</sup> liver sample was stored in formalin for 24 hours then in 70° ethanol at 4°C for hematoxylin/eosin and Sirius Red Staining.



**Figure 6. Steatosis, inflammation, fibrosis, hepatocyte and total NAS score at the end of treatment period.**

Inflammation and fibrosis largely contributed to the total NAS score, which was not changed with both CER-209 and obeticholic acid. A trend to decrease the steatosis was observed with CER-209 at 1 mg/kg dose.

## CONCLUSION

Recently, we demonstrated in vivo in mice that CER-209 acts as a key partner in HDL metabolism and the RLT process, and thereby promotes atherosclerosis protection. These data support a mechanism in which stimulation of HDL uptake or endocytosis by the liver, via activation of the P2Y13r pathway, promotes cholesterol catabolism by the liver, secretion of cholesterol in the gallbladder and elimination in the faeces. In the present study, we demonstrated that CER-209 lowers triglyceride and cholesterol concentrations in a new NAFLD/NASH model.

CER-209 treatment resulted in a decrease in the steatosis that is observed in diet induced mice model. As expected with such short-term treatment, the fibrosis and inflammation score was not changed; however, there was a tendency to decrease in the measured amount of steatosis. These findings were supported by the observation that cholesterol and triglyceride content of the livers also decreased with

CER-209 treatment. It is noteworthy that liver or animal weights were not observed to change with treatment in any of our animal studies.

An important observation is the strong decrease of the liver enzymes (ALT and AST) in the plasma due to CER-209 treatment. Those effects on the restoration of the liver integrity are in favor of a strong potential of CER-209 to treat liver disease such as NAFLD and/or NASH.

Finally, one can hypothesize that by increasing elimination of cholesterol (as bile acids and unesterified cholesterol) via the gallbladder, CER-209 may also trigger a decrease in triglycerides, which could have a beneficial effect on fatty liver. One of the mechanisms for the improvement in NASH with CER-209 may be an indirect increase in bile acid reabsorption by the intestine, promoting bile acid recycling to the liver. Indeed, an improvement in the hepatic triglyceride content following oral administration of bile acids was recently described in vivo in genetically obese mice (ob/ob mice) with NASH pathology.

P2Y13r is a new therapeutic target to treat atherosclerosis and NASH through a new mechanism of action. It is anticipated that CER-209 has a strong potential for treating the pathophysiology of Atherosclerosis and NASH due to its specific targeting of the pathways for cholesterol elimination, without the pleiotropic effects characteristic of drugs working through nuclear factors, such as PPAR and FXR agents.

## MATERIALS AND METHODS

After the acclimation period, mice (n=50) were put on a 60% high fat/1.25% cholesterol/0.5% cholic acid diet for 3 weeks. In order to promote hepatic cholesterol loading and liver complications under this specific diet, all mice were also treated with a 20% 2-hydroxypropyl beta-cyclodextrin aqueous solution by oral gavage, QD in the afternoon for 3 weeks.

At 1 week of diet, mice were bled at ~1:00pm to measure plasma ALT/AST and were randomized into 5 homogenous treatment groups (n=10/group) according to their 1) ALT, 2) AST and 3) body weight. Mice were then treated orally QD with vehicle, CER-209 at 3 different doses or obeticholic acid 25mg/kg for 2 weeks.

At 2 weeks of treatment, mice were weighed, treated at ~08:00am in the morning and bled (maximal volume/EDTA) at ~1:00pm. Whole blood sample were collected and plasma was then isolated (pellet was

stored at -80°C for eventual further analysis).

A 50µL-plasma sample was kept stored at -80°C prior to assay plasma ALT and AST. The plasma volume left over was separated into two 50µL-plasma aliquots and stored at -80°C prior to ALP plasma levels measurement and shipment of 75µL of plasma to Exiqon in Denmark. After blood collection, mice were sacrificed by cervical dislocation under isoflurane anesthesia and exsanguinated with sterile saline. For each mouse, liver was collected, a picture was taken and the liver was weighed.

A ~20mg liver sample was dissected for hepatic total cholesterol, triglycerides and fatty acids levels. A ~30mg liver sample was dissected and immediately flash frozen for gene expression analysis (optional).

A 0.5cm<sup>3</sup> liver sample was frozen in isopentane for oil red O staining. A 0.5cm<sup>3</sup> liver sample was stored in formalin for 24 hours then in 70° ethanol at 4°C for hematoxylin/eosin and Sirius Red Staining. The spare liver was kept stored at -80°C for eventual analysis.

As a significant effect was observed with the test item after biochemical analysis of plasma and liver samples, histology analysis and NAS scoring, as well as liver gene expression for a-SMA, collagen 1a1, TIMP1 and lumican analysis were performed.

Data are presented as mean ± SEM. A 1-way or 2-way ANOVA + Dunnett or Bonferroni post-test were used to perform statistical analysis. A p<0.05 was considered significant.