P2Y13 receptor agonist CER-209 decreases both atherosclerosis and liver steatosis *in vivo*

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Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are components of the growing worldwide epidemic of obesity. NAFLD is the most prevalent chronic liver disease, affecting 20%– 40% of the population, and approximately one third of patients with NAFLD will progress to nonalcoholic steatohepatitis (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. Insulin sensitizers such as the thiazolidinediones have been extensively tested, showing significantly reduced liver inflammation and steatosis but only modest efficacy in the control of liver fibrosis in patients with NASH. The use of the farnesoid X receptor (FXR), a member of the nuclear receptor superfamily which regulates a wide set of target genes involved in bile acid synthesis and transport, lipid metabolism and glucose homeostasis, may also be a promising strategy. The FXRs include pentoxifylline4, obeticholic acid (INT-747) and a dual peroxisome proliferator activated receptor (PPAR) agonist GFT505. Clinical trials of specific agents to treat NASH have begun to show promise. For example, there is partial consensus that vitamin E can be recommended in adults with advanced NASH who do not have diabetes or cirrhosis, although long-term follow-up data are not yet available3.

The treatment of dyslipidemia, which is frequently observed in patients with NAFLD, plays a critical role in the overall management for these patients. Statins are effective lipid-lowering agents (mainly through LDL-cholesterol), associated with a decrease risk of cardiovascular events in several interventional randomized clinical trials. The usefulness of statins for the treatment of NAFLD/NASH is still a matter of debate and randomized clinical trials of adequate size and duration are required. In contrast to the LDL metabolic pathway, HDL particles are accountable for reverse lipid transport (RLT), a concept proposed by Glomset and Wright as the overall flux of cholesterol from the entire periphery to the liver and its ultimate excretion. The capacity for HDL to increase bile acids and cholesterol elimination by the liver has never been studied in light of the NAFLD and NASH pathologies probably because one can hypothesize that the overall improvement of lipid elimination by the liver could impact fatty liver and steatohepatitis observed in patients.

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. In the present study, we first confirmed the increase of the number of HDL particles and their functionality, and thus the decrease of the atherosclerotic plaque development in a different atherosclerotic model, the high-cholesterol diet rabbit model. Importantly, we also observed for the first time CER-209 is able to lower triglyceride and cholesterol concentrations in the rabbit livers and to decrease the liver steatosis.

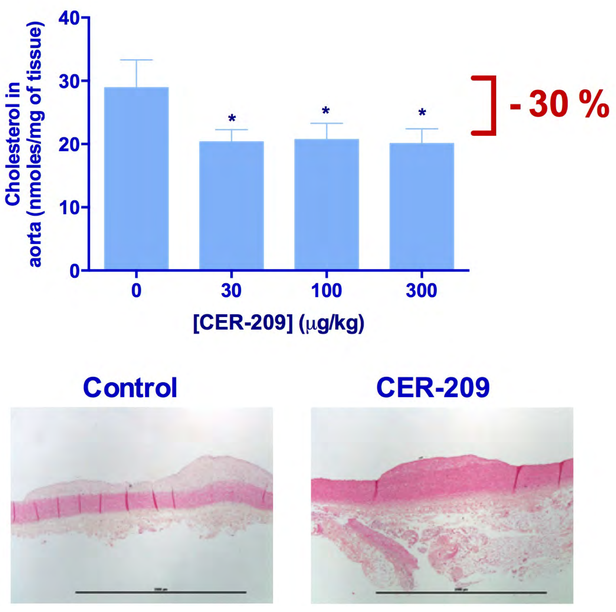


Figure 1: Plaque regression in rabbits treated with CER-209.
New Zealand rabbits (n = 15) with atherosclerotic plaques developed after 2 months of high-cholesterol diet, were treated with CER-209 at 30, 100 and 300 µg/kg once a day by oral gavage for 4 weeks. Lipids extracted from aortas were analyzed by GC/MS for cholesterol concentration.
*p < 0.05

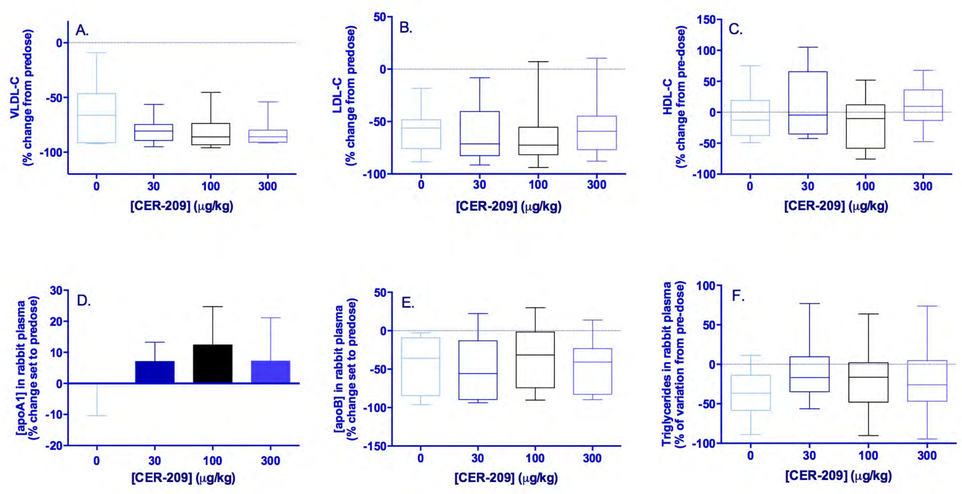


Figure 2: Lipid plasma parameters in rabbit treated with CER-209 or vehicle.
All results are expressed as the percentage change from pre-dose values. Panel A to C: Plasma VLDL cholesterol, LDL cholesterol and HDL cholesterol were measured as described in the Materials and Methods section. Panel D: apoA-I concentration in plasma was measured by SELDI-TOF analysis. Purified apoA-I from homo-sapiens (MWSELDI = 28083 Da) was used as a reference for determination of rabbit apoA-I (MWSELDI = 27838 Da) concentration. Panel E: apoB concentration in plasma was determined by Western blot analysis. Panel F: triglycerides measured in the plasma using a kit from Biolabo.

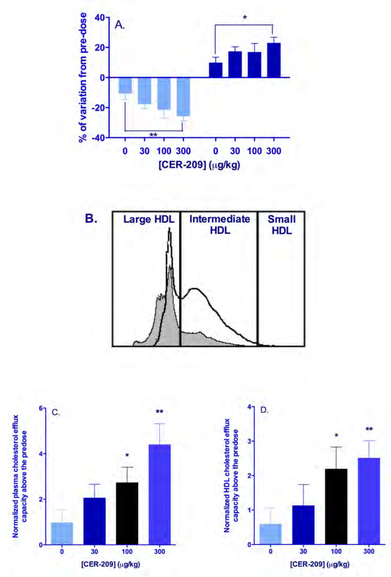


Figure 3: Size distribution and functionality of plasma from high-cholesterol diet fed rabbits treated with CER-209. Note the overall decrease in the HDL particle size of treated animals. Panel A: HDL from rabbit plasma was separated according to the size of the different HDL particles using the Lipoprint® system. The data were expressed as the percentage change for each HDL sub-population compared to the pre-dose HDL population in the animals. Two main HDL particle sub-populations were quantified (high and intermediate - dark and grey bars respectively). Panel B: example of lipoprotein profiles of rabbit treated with vehicle (grey line) and with CER-209 (300 µg/kg, dark line) using the Lipoprint® separation technique. Panels C and D: determination of cholesterol efflux capacity of plasma and plasma apoB-depleted respectively using pre-loaded [3H]-cholesterol-oxLDL macrophages. The results are expressed as a percentage of cholesterol efflux corrected from pre-dose. Values for cholesterol efflux before correction from pre-dose vary from 3 to 6%.

Regression of Atherosclerosis after CER-209 treatment in HFD rabbit model was confirmed.

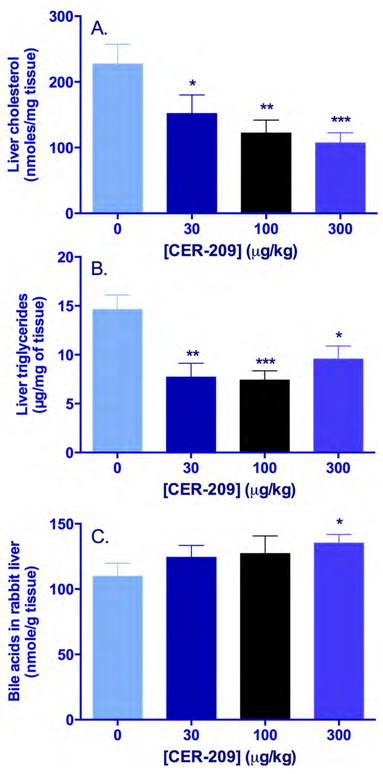
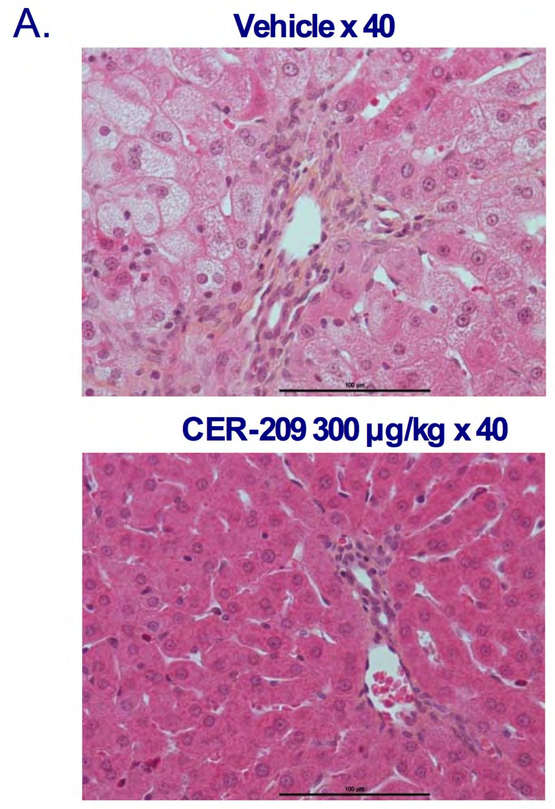


Figure 4: Decrease of liver triglyceride and cholesterol concentrations in high-cholesterol diet rabbits treated with CER-209. Livers were analyzed for total cholesterol (Panel C) and triglyceride (Panel A) contents using gas- chromatography (GC). Panel B: The bile acid concentrations in livers were determined using an enzymatic kit.
*p < 0.05, **p < 0.005, ***p < 0.0005.



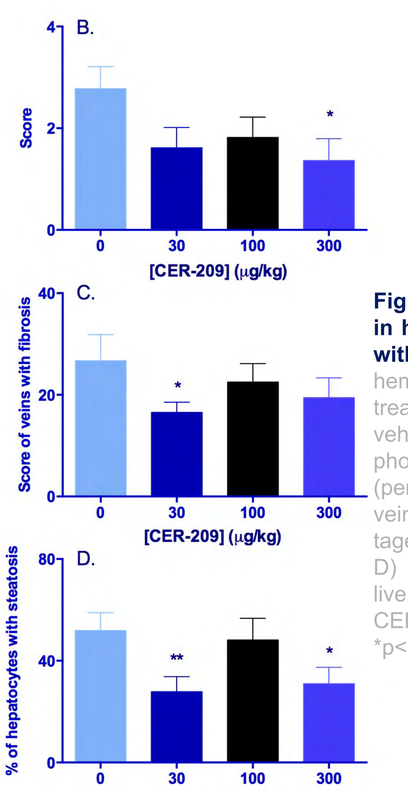


Figure 5: Regression of liver steatosis in high-cholesterol diet rabbits treated with CER-209. Panel A: Representative hematoxylin-eosin staining of livers from treated animals (0.3 mg/kg) compared to vehicle control group. Panels B to D: morphological quantification of steatosis (perilobular and centrilobular - Panel B), of vein fibrosis (Panel C) and of the percentage of hepatocytes with steatosis (Panel D) after hematoxylin-eosin staining of livers from treated animals at different CER-209 doses.
*p<0.05, **p<0.01.

CONCLUSION

CER-209, a P2Y13 receptor agonist, is able to induce an increase in the number of circulating small HDL particles and to lower large HDL particles by a yet unknown mechanism. Using a P2Y13r agonist would increase the clearance of the large HDL particles by the liver and favor the reverse lipid transport. Indeed, it has already been described in the literature that mature large HDL particles preferentially interact with hepatocytes compared to smaller HDL particles.
The overall implication of this increase is to allow not only the removal of cholesterol from atherosclerotic plaques, but also to regulate lipid homeostasis in the liver. It has previously been demonstrated that the behavior of cholesterol coming from HDL is different from cholesterol coming from the ß-lipoprotein (i.e. VLDL and HDL). The cholesterol transported to the liver by HDL follows a pathway with direct elimination of cholesterol in the bile acid flux and also a second indirect but perhaps more important role, as a main substratum for bile acid synthesis. This is the only mechanism allowing cholesterol to be eliminated from the body.
One can hypothesize that by increasing the cholesterol elimination into the gallbladder as bile acids and unesterified cholesterol we will also trigger a decrease in triglycerides which could have a beneficial effect on fatty liver. One of the mechanisms for the improvement in NASH could be the indirect increase in bile acid reabsorption by the intestine. Indeed, an improvement in the hepatic triglyceride content following oral administration of bile acids was recently described in vivo in obese mice (ob/ob mice) with NASH pathology.

Other molecules designed to improve the NASH, other than CER-209, have been described. For instance, insulin sensitizers such as the thiazolidinediones have been extensively tested, showing significantly reduced liver inflammation and steatosis but unfortunately only modest efficacy in controlling liver fibrosis in patients with NASH. The targeting of the FXR, a member of the nuclear receptor superfamily which regulates a wide set of target genes involved in bile acid synthesis and transport, lipid metabolism and glucose homeostasis, seems to be a promising strategy. Obeticholic acid (Intercept) is currently in Phase II clinical trials for NASH. Finally, a dual peroxisome proliferator activated receptor agonist GFT505 (Genfit) has been also targeted to NASH pathology by its pleiotropic effect on metabolic syndrome.

P2Y13r is a new therapeutic target with a new mechanism of action. It is anticipated that CER-209 has a strong potential for treating the pathophysiology of 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

- **Animal protocol.** Animal housing and care were in compliance with the recommendations of Directive 86/609/EEC, and protocol approvals were obtained from institutional ethics committees.
- **New Zealand rabbit diet.** New Zealand rabbits (4-5 week old) were fed with High Cholesterol Diet (0.5 % cholesterol) for 2 months followed by a wash-out period of 2 weeks. The animals were then dosed once a day with compound CER-209 at 30, 100 and 300 µg/kg for 4 weeks. Blood samples were collected before the dose administration on day 0 and after 4 weeks of administration of CER-209. After final bleeding, the liver, gallbladder, heart, thoracic and abdominal aorta from heart (left ventricle) to iliac arteries were sampled.
- **Aorta cholesterol measurement.** The samples were lipid extracted overnight at 4°C in CHCl3:MeOH (2:1) with addition of stigmasterol as internal standard. Samples were assayed for total cholesterol after saponification in methanolic KOH and analyzed by GC/MS (Synelvia, Labege, France).
- **Plasma lipoproteins profiles.** Lipoproteins profiles were measured by HPLC using a Sepharose 6 column and detected for total cholesterol with inline enzymatic detection2 (Synelvia, Labege France).
- **SELDI-TOF analysis.** Rabbit plasma (1:50 dilution) were supplemented with apoA-I from homo-sapiens (1:1 apoA-Ihs:apoA-Ioc) as internal reference for determination of the rabbit apoA-I concentration. ProteinChip Q10 was used as the matrix. The experiment was conducted by the protein profiling platform of IFR150, Toulouse, as previously described.
- **ApoB100 analysis in rabbit plasma.** Rabbit plasma (1µl) was separated on 6% SDS-PAGE and transferred onto PVDF membranes. ApoB protein was detected with anti-apoB antibody (ROCKLAND, goat antibody) and the bands were quantified by Image-J software. The surface area for each band in the post-dose groups was compared to their respective pre-dose.
- **Plasma triglyceride determination.** Concentrations were determined according to the manufacturer protocol with enzymatic kit from Biolabo (Biolabo – France).
- **Lipoprint profiles.** Rabbit plasma (25µL) was analyzed with the Lipoprint HDL system (Quantimetrix corporation) according to the manufacturer protocol.
- **Cholesterol efflux determination.** Cholesterol efflux capacity was quantified in blood samples from rabbit plasma collected before administration of the dose on day 0 and after 4 weeks of administration of CER-209 as previously described.
- **Rabbit liver staining.** Slices of rabbit livers were fixed in neutral formalin buffer and embedded in paraffin. The determination of steatosis (200 hepatocytes/rabbit) and fibrosis (12 veins/rabbit) was performed after HES coloration.
- **Liver cholesterol and triglyceride measurement.** Liver (5-10mg) was homogenized in 2mL of methanol/ 5mM EGTA (2:1 v/v) with FAST-PREP (MP Biochemicals). The equivalent of 0.5mg of tissue was evaporated, the dry pellets dissolved in 0.25 mL of NaOH (0.1M) overnight and proteins were measured with the Bio-Rad assay. Lipids corresponding to 1mg of homogenized tissue were extracted (Bligh and Dyer method) using stigmasterol, 1,3-dimyristine, cholesteryl heptadecanoate and glyceryl trinonadecanoate as internal standards. Extracts were analyzed by gas-liquid chromatography on a FOCUS Thermo Electron system using Zebron-1 Phenomenex fused silica capillary columns (5m X 0,32mm i.d, 0.50 µm film thickness) as previously described. (Lipidomic platform, INSERM IFR150-Metatoul, Toulouse).
- **Bile acid measurement.** Bile acid concentration was determined with the Diazyme kit and validated by comparison with HPLC method. 50 mg of liver sample were extracted with PBS and analyzed with the Diazyme kit.
- **Rabbit QPCR.** RNA from liver samples were extracted with the Ribopure kit (Ambion) and reverse transcripted with the High capacity RNA to cDNA kit (Applied Biosystems) according to the manufacturer protocols. Real time quantitative PCR of apoA-I was performed with taqman probe (Oc03399269.g1, Applied Biosystems).

Statistical analysis. All statistical analysis were performed using T-test protocol with 95% confidence using Prism software (Graphpad).