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Abstract

Background : Atherosclerosis and non-alcoholic fatty liver disease (NAFLD) are complex pathologies characterized by lipid accumulation, chronic inflammation and extensive tissue remodelling. Microparticles (MPs), small membrane vesicles produced by activated and apoptotic cells, might not only be biomarkers, but also functional actors in these pathologies. The apoE2-KI mouse is a model of atherosclerosis and NAFLD. Activation of the nuclear receptor PPARα decreases atherosclerosis and components of non-alcoholic steatohepatitis (NASH) in the apoE2-KI mouse.

Objectives : 1) To determine whether MPs are present in atherosclerotic lesions, liver and plasma during atherosclerosis and NASH progression in apoE2-KI mice, and 2) to study whether PPARα activation modulates MP concentrations.

Methods : ApoE2-KI mice were fed a Western diet to induce atherosclerosis and NASH. MPs were isolated from atherosclerotic lesions, liver and blood and quantified by flow cytometry.

Results : An increase of MPs was observed in the atherosclerotic lesions and in the liver of apoE2-KI mice upon Western diet feeding. PPARα activation with fenofibrate decreased MP levels in the atherosclerotic lesions in a PPARα-dependent manner, but did not influence MP concentrations in the liver.

Conclusion : Here we report that MPs are present in atherosclerotic lesions and in the liver of apoE2-KI mice. Their concentration increased during atherosclerosis and NASH development. PPARα activation differentially modulates MP levels in a tissue-specific manner.

Keywords : microparticles, atherosclerosis, fatty liver disease, pharmacology, murine model

Introduction

The prevalence of metabolic syndrome associated with visceral obesity is in constant progression world-wide [1, 2], resulting in a high risk of developing complications such as diabetes and cardiovascular diseases. These pathologies are pathophysiologically related to atherosclerosis and non-alcoholic fatty liver disease (NAFLD), including non-alcoholic steatohepatitis (NASH), which display common features such as lipid accumulation and inflammation [3, 4]. They develop silently over several years, and are the most common causes of cardiovascular and chronic liver diseases. Consequently, early diagnosis of atherosclerosis and NASH is crucial to allow early intervention and prevent disease progression. Therefore, the discovery of biomarkers is an important approach for the diagnosis and prognosis of these diseases.

Atherosclerosis and NASH progression are both accompanied by inflammation development which involves several actors including different chemokines (monocyte chemoattractant protein 1, MCP-1), cytokines (tumor necrosis factor, TNF) and metalloproteinases (MMP) [5-8]. Tissue remodelling and inflammation, occurring during atherosclerosis or NAFLD, may induce microparticle (MP) formation. MPs are small vesicles (0.1-1µm) released from cells undergoing apoptosis or under stress conditions such as inflammation. MPs expose phosphatidylserine in the outer leaflet of their membrane, allowing their quantification by flow cytometry after staining with annexin V coupled to a fluorophore [9]. MPs are present at relatively low concentrations in the circulation of healthy individuals. Numerous clinical studies have reported increased plasma MP levels associated with cardiovascular risk factors (hypertension, smoking, obesity and type 2 diabetes) and cardiovascular diseases [10-15]. Moreover, MPs are also present in human atherosclerotic lesions [16]. Concerning liver diseases, an increase of plasma MPs has been reported in patients with hepatitis C [17] and hepatocellular carcinoma [18], but it is unknown whether

liver MPs are detectable in patients with NASH. MPs display several functional properties that can influence atherosclerosis and NASH development, such as induction of endothelial dysfunction, angiogenesis modulation, inflammation exacerbation and thrombus formation [19-22]. Furthermore, several drugs used in the treatment of cardiometabolic diseases, such as statins and glitazones, reduce plasma MP concentrations [23, 24]. Thus, MPs might be considered as disease biomarkers, and may also be useful to predict drug efficacy in preclinical and early clinical development. However, experimental evidence in preclinical models on the role of MPs in the pathological processes *in vivo* is still lacking. Such experiments are necessary to further validate MPs as potential disease biomarkers.

Peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear receptor, activated by fatty acids and synthetic ligands such as the hypolipidemic fibrates, which regulates the expression of genes implicated in lipid homeostasis and inflammation [25]. PPARα agonists inhibit atherosclerosis lesion development in preclinical models[26-28], and are of particular interest for the treatment of cardiometabolic diseases, especially in patients exhibiting high triglyceride (TG) and low high density lipoprotein cholesterol (HDL-C) levels [29, 30]. By contrast, the effects of fibrates on NASH in humans have not been thoroughly studied.

The apolipoprotein E2 knock-in (apoE2-KI) mouse is an experimental model of atherosclerosis and NASH [26, 31]. These mice develop spontaneously dyslipidaemia and atherosclerotic lesions in the aortic sinus, essentially characterized by foam cell accumulation. Treatment with the PPARα activator fenofibrate improves dyslipidaemia and reduces atherosclerosis development [26]. Moreover, short-time Western diet feeding is sufficient to induce hepatic steatosis and inflammation, effects which can be prevented by fenofibrate treatment [31].

The aim of the present study was to determine in apoE2-KI mice whether 1) liver and aortic MPs can be detected as markers of disease progression, 2) plasma MPs are diagnosis markers of pathology, and 3) to analyse whether MP concentrations can be pharmacologically modulated by PPARα activation. Our results show an increase of MP concentrations in atherosclerotic lesions and liver of apoE2-KI mice upon Western diet feeding. Fenofibrate treatment decreased MP levels in the atherosclerotic lesions in a PPARα-dependent manner, but did not influence NASH-associated MP concentrations.

Methods

Animals and diets

Homozygous human apoE2-KI mice, which express human apoE2 in the mouse apoE locus, were used in this study [32]. For some protocols, homozygous PPARα-deficient mice on the C57BL/6 background [33] were crossed with apoE2-KI mice, to generate apoE2-KI mice deficient for PPARα and their littermates expressing PPARα.

Twelve-week old female mice were fed a chow diet or a Western diet (21% fat and 0.2% cholesterol) (Safe, France) supplemented or not with fenofibrate (0.04% for 10 weeks or 0.2% for 2 weeks). For NASH studies, the Western diet was supplemented or not with fenofibrate 0.2% for 10 days. Doses and treatment durations were chosen according to their inhibiting effects on atherosclerotic lesion and NASH development, respectively [26, 31]. All experiments were performed with 8 animals per group.

Blood was obtained after a 4-hour fasting period (9 AM to 13 PM) by retroorbital puncture under isoflurane-induced anesthesia, and was collected either on EDTA-coated tubes for plasma lipid measurements or in tubes containing trisodium citrate 3.2% at 1 : 10 vol of blood for MP quantification.

Mice were maintained under a 12 hour light/dark cycle and had free access to food and water. All animal experiments were performed with the approval of the Pasteur Institute review board, Lille, France.

Biochemical analysis

Plasma levels of total cholesterol (TC), TGs and HDL-C were measured using commercially available kits (BioMérieux, France). Non-HDL-cholesterol (N-HDL-C) was calculated by subtraction of HDL-C from TC.

MP isolation from tissues

Fresh tissues (the aortic sinus-containing part of the heart or the liver) were weighed, rinsed in PBS solution and homogenized in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 10 µL/mg tissue). MP isolation protocol from tissues was adapted from Leroyer et al.[16]. Briefly, samples were finely cut and centrifuged at 400g, 15 minutes at 4°C followed by 12500g, 5 minutes at 4°C to remove cells and cellular debris. Supernatants were subsequently centrifuged at 20000g, 45 minutes at 4°C to sediment MPs. MP pellets were then resuspended in DMEM (10µL medium/mg tissue). Samples were stored at -80°C until MP quantification.

Platelet-free plasma (PFP) preparation

Citrate-collected blood was centrifuged at 1500g, 15 minutes at 20°C to remove blood cells and then at 13000g, 2 minutes at 20°C to remove platelets. Platelet-free plasma (PFP) was immediately frozen in liquid nitrogen and stored at -80°C until MP quantification.

MP quantification by flow cytometry

MP samples (PFP or MPs isolated from tissues) were incubated with fluoroisothiocyanate-conjugated annexin V (BD Biosciences, France) according to the manufacturer's instructions. MP analysis and quantification were performed using calibrated 10µm-sized beads of known concentration on a FC 500 cytometer (Beckman Coulter, France). MP population was determined on a gate of 0.1-1 µm events on a forward light scatter and side light scatter dot plot representation, and as positive events on fluorescence/forward light scatter plot as previously described [34]. Results are expressed as percentage of MP concentration relative to the control group set as 100%. For specific cellular origin determination, the following specific antibodies and their specific isotype controls were used: CD68 coupled to phycoerythrin (PE) for monocytes and F4/80 coupled to phycoerythrin-cyanin 5 (PCy5) for macrophages, CD144-PE for endothelial cells, TER119-PE for erythrocytes, CD41-PCy5 for platelets. All antibodies were purchased from BD Bioscience (France) and used at 20 ng/mL, except CD68 which was from Beckman Coulter (France) and used at 10 ng/mL.

Analysis of atherosclerotic lesions

After sacrifice by cervical dislocation, hearts were fixed with 4% phosphate-buffered paraformaldehyde (PAF) and 10µm-aortic sinus sections were cut followed by quantitative analysis of lipid deposition by Oil red-O staining. Sections were also stained with rat monoclonal anti-mouse macrophage MOMA-2 (Santa Cruz Biotechnology, Germany), followed by detection with biotinylated secondary antibody and streptavidin-horseradish peroxidase. Images were captured using a JVC 3-charge-coupled device video camera and analysed using the computer-assisted Quips Image analysis system (Leica Mikroscopic und System Gmbh, Germany).

Hepatic lipid analysis

Frozen liver tissue (50 mg) was homogenized in SET buffer (1 mL ; sucrose 250 mM, EDTA 2 mM and Tris 10 mM), followed by two freeze-thaw cycles and three times passing through a 27-gauge syringe needle and a final freeze-thaw cycle. Protein content was determined using the BCA method (Interchim, France) and TG and cholesterol measured as described above.

Histological analysis of the liver

At sacrifice, livers were perfused with phosphate buffered saline (PBS) solution via the portal vein. After removal of the liver, a part of about 4 mm^2 was fixed in PAF and embedded in paraffin. Five µm paraffin-embedded sections were stained with haematoxylin/eosin to evaluate steatosis and inflammation. Seven µm of frozen-liver sections were stained with rat monoclonal anti-mouse macrophage MOMA-2 (Santa Cruz Biotechnology, Germany), followed by detection with biotinylated secondary antibody and streptavidin-horseradish peroxidase, to evaluate macrophage content. Analysis of lipid deposition was made by Oil red-O staining on seven µm of frozen-liver sections, using Harris haematoxylin for nucleus coloration.

RNA analysis

Total RNA was isolated from frozen tissues using the acid guanidium thiocyanate/phenol/chloroform method. RNA was reverse-transcribed using Moloney murine leukaemia virus-reverse transcriptase and random hexamer primers (Invitrogen, France). mRNA levels were quantified by real-time quantitative PCR on a MX-3000 apparatus (Agilent, France) using the Brilliant SYBR Green QPCR master mix (Stratagene) and specific primers (Table I). Results are expressed normalized to cyclophilin mRNA.

Statistical analysis

The significance of differences (mean ± sem) between groups was determined by Mann&Whitney test when the analysis was made on 2 groups and ANOVA analysis followed by between group post-hoc analysis using the Scheffe test when the analysis was made on 4 groups. A value of $p<0.05$ was considered as statistically significant.

Results

MP concentrations are elevated in the aortic sinus and in the liver of apoE2-KI mice with atherosclerosis and NASH.

To induce atherosclerotic lesion development, apoE2-KI mice were fed a Western diet for 10 weeks [26]. MP concentrations in the aortic sinus and in PFP were compared to apoE2-KI mice fed a chow diet. As expected, compared to mice fed a chow diet, mice fed a Western diet displayed an aggravated dyslipidaemia with increased plasma TC and TG levels (Sup figure 1), and accelerated lesion development in the aortic sinus (figure 1A). In chow diet fed mice, MP concentrations were 699±77 MPs/µL in the aortic sinus and 153±46 MPs/µL in PFP. Interestingly, MP concentrations in the aortic sinus were significantly higher in mice fed a Western diet compared with mice fed a chow diet (figure 1B). By contrast, PFP-MP concentrations were similar between the two groups (figure 1C). To better characterize the cell origin of the MPs, specific markers were analyzed on MPs isolated from atherosclerotic lesions and PFP. As in humans, PFP-MPs mainly originate from platelets and erythrocytes. Since macrophages and endothelial cells are the main cells composing atherosclerotic lesions of apoE2-KI mice, the concentrations of endothelial- and macrophage-derived MPs were measured in the atherosclerotic lesions, and platelet-, erythrocyte- and monocyte-derived MPs were measured in the PFP of mice fed a Western diet (Sup figure 2).

In the atherosclerotic lesions, 18% of MPs were positive for the macrophage marker F4/80 and 35% positive for the endothelial marker CD144. In PFP, 73% of MPs were positive for the erythrocyte marker TER119 and 4% positive for the platelet marker CD41, whereas no MP positive for the monocyte marker CD68 was detectable on our conditions.

To induce NASH, another group of apoE2-KI mice were fed a Western diet for 10 days [31]. Western diet feeding increased hepatic TG levels (figure 1D) and strongly increased in TNF and MCP-1 gene expression in the liver (figure 1E-F). Metalloproteinase-9 (MMP-9) gene expression showed a tendency to increase (figure 1G). In chow diet fed mice, MP concentrations were 8054±2196 MPs/µL in the liver and 96±20 MPs/µL in PFP. Liver MP concentration increased in mice fed a Western diet (figure 1H). However, PFP-MP concentrations were not significantly different between the mice fed a chow diet and the mice fed a Western diet (figure 1I). Macrophage-derived MP concentration in the liver and platelet-, erythrocyte- and monocyte-derived MP concentrations in the PFP were measured in mice fed a Western diet (Sup figure 2). In the liver of these mice, 3% of MPs were positive for the macrophage marker F4/80. In PFP, 88% of MPs were positive for the erythrocyte marker TER119 and 12% positive for the platelet marker CD41, whereas no MP positive for the monocyte marker CD68 was detectable on our conditions.

Inhibition of atherosclerotic lesion development by fenofibrate is associated with a decreased MP concentration in the aortic sinus and PFP.

Considering that fenofibrate treatment inhibits atherosclerosis progression in apoE2-KI mice, MP levels were determined in mice fed a Western diet supplemented or not with fenofibrate (0.04 %) for 10 weeks. Fenofibrate treatment increased expression of the PPARα target gene acyl-coA oxidase (ACO) (Sup figure 3A), decreased plasma TC and non-HDL-C concentrations and increased plasma HDL-C levels (Sup figure 3B). As previously

shown [26], fenofibrate treatment inhibited atherosclerosis lesion progression (figures 2A, 2B), and under these conditions, also significantly reduced MP concentrations in the aortic sinus (figure 2C) and in PFP (figure 2D). To determine whether these effects were dependent on PPARα, experiments were performed in apoE2-KI mice deficient for PPARα, in which fenofibrate has no effect on dyslipidemia and atherosclerosis development (our unpublished data). Treatment of these mice with fenofibrate did not influence MP concentrations neither in the aortic sinus or PFP (figures 2C, 2D).

Short-term fenofibrate treatment decreases MP content in the aortic sinus of mice with established atherosclerotic lesions.

To determine whether PPARα activation exerts its effects on MP concentrations independently of changes in plaque size, apoE2-KI mice were fed a Western diet for 8 weeks in order to induce atherosclerotic lesion development, and subsequently treated or not with fenofibrate (0.2%) for two additional weeks. Fenofibrate treatment increased ACO expression (Sup figure 4A), and reduced the Western diet-induced dyslipidaemia as assessed by TC, non-HDL cholesterol, TG and HDL-C concentration changes (Sup figure 4B). Under these conditions of short-term treatment, fenofibrate did not significantly influence atherosclerosis lesion area, nor lipid and macrophage content of the atherosclerotic lesions (figure 3A-C). Moreover, gene expression of apoptosis markers, such as B-cell lymphoma-2 (Bcl-2), caspase-3 (casp-3) and Bcl-2 associated protein (BAX) was not modified by the treatment. By contrast, expression of the inflammatory response genes MCP-1, interleukin-6 (IL-6) and TNF was significantly reduced in the aortic sinus of fenofibrate-treated mice (figure 3D). The expression of genes involved in efferocytosis, such as thrombospondin-1 (TSP-1), growth arrest specific-6 (GAS-6) and c-mer proto-oncogene tyrosine kinase (MERTK) was not modified in the aortic sinus upon fenofibrate treatment (figure 3E).

Interestingly, fenofibrate reduced MP concentrations in the aortic sinus (figure 3F), whereas PFP-MP concentrations were not significantly modified (figure 3G). The fenofibrate-induced decrease of MP levels was not observed in apoE2-KI mice deficient for PPARα (figure 3F), indicating that the effect is dependent on PPARα. In atherosclerotic lesions of fenofibrate-treated wild-type mice, the proportion of endothelial-derived MPs was not modified (35.2±0.8% versus 34.5±0.5%, treated group versus control group), whereas the proportion of macrophage-derived MPs was significantly reduced (5.6±1.7% versus 18.4±0.8%, treated group versus control group, $p<0.001$).

Fenofibrate does not influence liver or PFP-MP concentrations in apoE2-KI mice.

To assess the effect of fenofibrate in a model of NASH, apoE2-KI mice were fed a Western diet supplemented or not with fenofibrate 0.2% for 10 days [31]. Fenofibrate treatment led to the expected effects on hepatic ACO gene expression (Sup figure 5A) and improved the Western diet-induced dyslipidaemia (Sup figure 5B). Moreover, fenofibrate treatment reduced the hepatic lipid content (figure 4A), and the expression of genes involved in inflammatory response (figure 4B) and efferocytosis (figure 4C). Treatment with fenofibrate improved steatohepatitis as shown by haematoxylin/eosin, MOMA-2 and Oil Red O staining of livers (figure 4D). However, in these conditions, treatment with fenofibrate had no effect on MP concentrations neither in the liver (figure 4E) nor in PFP (figure 4F).

Discussion

Atherosclerosis is characterized by lipid accumulation, chronic inflammation and extensive tissue remodelling [35], conditions favourable to trigger MP production by cells of the vascular wall, including macrophages, endothelial cells and smooth muscle cells. Interestingly, MPs have been detected in atherosclerotic plaques of human carotid arteries

[16]. The apoE2-KI mouse fed a Western diet is a model of early atherogenesis [32] in which atherosclerotic lesions are mainly composed of foam cells resembling fatty streaks in humans. In the present study, we have shown the presence of MPs in a mouse model of atherosclerosis and that progression of atherosclerosis in apoE2-KI mice increases MP concentrations in the aortic sinus. Steady-state MP concentrations reflect the equilibrium between MP release by apoptosis and/or inflammatory stimuli and MP clearance by phagocytic cells. As apoE2-KI mice display very low levels of apoptosis in the aortic sinus (unpublished data), MPs in the plaques of these mice are likely the result of the inflammation associated with lesion development. In addition, since MPs expose phosphatidylserine, an eat-me signal for macrophage recognition and phagocytosis [36], MPs could be eliminated by processes similar as for apoptotic cells, namely efferocytosis [37, 38]. Thus the increase of MPs in plaques could be due to an imbalance between increased production by cells and/or decreased elimination by phagocytes.

Numerous studies reported an association between plasma MP concentrations and cardiovascular risk factors [10-15]. Therefore, MPs are potentially interesting biomarkers, both as diagnosis and prognostic markers. In our model, plasma MP concentrations did not increase during atherosclerosis in apoE2-KI mice. However, plasma MP concentrations in this model are very low (in the range of 200-300 MPs/µL) compared to those in atherosclerotic patients [16] or in other murine models of atherosclerosis such as low density lipoprotein-receptor (LDL-R)-deficient mice fed a Western diet for 20 weeks [39]. Moreover, in apoE-deficient mice, PFP-MP concentrations are higher in 19 compared to 4 week old-mice (363±151 MPs/µL versus 176±50 MPs/µL, our own observations). As apoE-deficient mice develop severe atherosclerosis with age associated with a relatively small increase in MP concentration, it is possible that the lack of an increase in plasma MP concentrations in apoE2-KI mice under Western diet is due to the relatively mild atherosclerosis development.

Previous studies have established that fenofibrate treatment reduces atherosclerosis in apoE2-KI mice by reducing both lipid content and inflammation in the aorta [26]. In this model, the effects of fenofibrate on plasma lipid concentrations are similar to those observed in humans, namely TG reduction and HDL-C increase, while PPARα activation has different effects in other atherosclerosis models such as apoE-deficient mice or LDL-R-deficient mice [28, 40]. Thus, the apoE2-KI mouse is an appropriate pharmacological model to study the effects of PPARα activation on dyslipidemia and atherosclerosis. In the present study, fenofibrate treatment reduced MP concentrations in the aortic sinus, in association with reduced lesion area and inflammation. This effect did not occur in apoE2-KI PPARα-/- mice, proving PPARα-dependency. Interestingly, fenofibrate treatment decreased PFP-MP concentrations, which possibly reflects the whole body anti-inflammatory effects of fenofibrate. Moreover, short-term treatment of apoE2-KI mice with established lesions, in which fenofibrate did not reduce atherosclerotic lesion area nor lipid and macrophage content in the aortic sinus, resulted in decreased lesion MP concentrations. This suggests that the effect of PPARα activation on MP content is independent of its effect on plaque size and composition. Short-term treatment with fenofibrate reduced inflammation in the aortic sinus, as assessed by decreased expression of genes encoding inflammatory cytokines and chemokines, which are known stimuli of MP production. Conversely, since MPs are potent inducers of the inflammatory response, reduction of their concentration can contribute to the local anti-inflammatory action of fenofibrate. In the aortic sinus, fenofibrate treatment did not influence the expression of genes involved in efferocytosis, suggesting that PPARα activation has no effect on MP clearance in the aortic sinus. Consequently, PPARα activation could inhibit plaque progression by reducing production of MPs, stimuli known to be involved in cell recruitment and lesion destabilization.

Despite the fact that the mechanisms involved in NASH development are not clearly known, it is commonly accepted that it is a progressive process involving steatosis and inflammation [41]. It is reasonable to expect that these conditions may result in MP generation by liver cells, such as the hepatocytes, Kupffer cells and/or recruited macrophages. There are no clinical data available linking MPs with fatty liver diseases. The apoE2-KI mouse fed a Western diet for 2 weeks is a model of early NASH, characterized by moderate steatosis and inflammation in the liver [31]. In our study, we have shown that NASH progression in apoE2-KI mice results in an increase of MPs in the liver, which could in turn exacerbate the pathology.

The diagnosis of NASH is difficult in humans, because the pathology is usually asymptomatic and no specific circulating markers have been identified so far. Liver disease is determined by high liver transaminase levels in plasma. Imaging techniques are used to quantify lipid accumulation, but they do not allow inflammation or fibrosis detection. The most informative way to diagnose NASH is by performing a liver biopsy, which allows histological analysis and classification according to disease severity. However, this technique is invasive, and consequently difficult to perform on all patients. Hence, a link between PFP-MP and liver disease could make MPs a valuable marker of NASH, in the same manner that MPs are increased in hepatitis [17, 18]. In our study, we did not observe any variation of total plasma MP concentrations during NASH development. This can be explained by early development of NASH in this model, which may be not sufficient to induce an increase in plasma MP concentrations or the fact that PFP-MPs are mainly of erythrocyte origin. Mouse models of severe liver disease could provide information whether PFP-MP concentrations increase in more advanced pathology.

Fenofibrate decreases lipid content, steatosis and inflammation in livers of apoE2-KI mice fed a Western diet [31], but has no effect neither on hepatic nor on PFP-MP levels.

Despite the fact that fenofibrate treatment reduces liver inflammation and macrophage number, stimuli responsible for MP production, it also decreases expression of genes involved in efferocytosis, potentially leading to impaired clearance of MPs. Indeed, fenofibrate treatment could reduce MP production, but also decrease MP clearance, resulting in unchanged MP concentrations.

We furthermore determined the cellular origin of MPs using specific antibodies. Surprisingly, we found that the proportion of MPs originating from macrophages is low in atherosclerotic lesions and liver (18% and 3% respectively). Atherosclerotic lesions of apoE2-KI mice are mainly composed of macrophages and this cell type represents about 10% of total cells in the liver. One explanation for the relatively low proportion of macrophage-derived MPs may be that cell-specific markers at the surface of macrophages could be heterogeneously transferred to MPs. Hence, MPs produced by blebbing from macrophages may not express all markers from the cell of origin. For example, only 20% of MPs obtained *in vitro* from the RAW264.7 mouse macrophage cell line after stimulation with actinomycin D (0.5µg/mL) for 24 hours express the macrophage markers F4/80 or CD68.

Conclusion

In conclusion, the apoE2-KI mouse is an appropriate preclinical mouse model to study tissue MPs as markers of atherosclerosis and NASH. In this model, PPARα activation modulates MP concentrations in atherosclerotic lesions but not in the liver, identifying this model to be appropriate for pharmacological testing using different pharmacological approaches.

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Figure Legends

Figure 1 : Atherosclerosis and NASH progression are associated with increased MP concentration in atherosclerotic lesions and in liver but not in PFP of apoE2-KI mice.

ApoE2-KI mice were fed a chow or Western diet for 10 weeks to induce atherosclerosis development (A,B,C) or for 10 days to induce NASH development (D,E,F,G,H,I). Atherosclerotic lesion area was quantified by Oil Red O staining (A). Steatohepatitis was evaluated measuring liver lipid content (D) and inflammatory gene expression (E,F,G). MPs were extracted from aortic sinus (B), liver (H) and platelet-free-plasma (C, I) and quantified.

Statistically significant differences are indicated (Mann&Whitney test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 2 : Fenofibrate decreases atherosclerotic lesion-associated and PFP-MP concentrations in a PPARα dependent manner in apoE2-KI mice.

ApoE2-KI PPARα +/+ and -/- mice were fed for 10 weeks a Western diet supplemented or not with fenofibrate (0.04%). Atherosclerotic lesion area was quantified after Oil Red O staining (A,B). MPs were extracted from the aortic sinus (C), platelet-free-plasma (D) and quantified.

Statistically significant differences are indicated (A : Mann&Whitney test, * $p<0.05$; C,D,E : ANOVA test, * $p<0.05$, ** $p<0.01$).

CON : Control group, FF : Fenofibrate-treated group

Figure 3 : Short-time PPARα activation decreases MP content in developed atherosclerotic lesions, but has no effect on PFP-MP concentrations in apoE2-KI mice.

ApoE2-KI PPARα +/+ and -/- were fed for 8 weeks a Western diet alone and then with a Western diet supplemented or not with fenofibrate (0.2%) for 2 additional weeks. Atherosclerotic lesion area (A) and lipid content (B) were quantified after Oil Red O staining. Macrophage content was measured by MOMA-2 staining (C). Expression of genes involved in inflammation, apoptosis (D) and efferocytosis (E) was measured in the aortic sinus. MPs were extracted from the aortic sinus (F) and platelet-free-plasma (G) and quantified.

Statistically significant differences are indicated (ANOVA test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

CON : Control group, FF : Fenofibrate-treated group

Figure 4 : Fenofibrate treatment does not influence MP concentration in liver and in PFP of apoE2-KI mice.

ApoE2-KI mice were fed for 10 days with Western diet supplemented or not with fenofibrate (0.2%). Hepatic steatosis was evaluated by measuring lipid content (A), inflammatory gene expression (B), haematoxylin/eosin (H&E), MOMA-2 and Oil Red O staining (D). Expression of genes involved in efferocytosis was measured in the liver (C). MPs were extracted from liver (E) and platelet-free-plasma (F) and quantified.

Statistically significant differences are indicated (Mann&Whitney test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

CON : Control group, FF : Fenofibrate-treated group