MIRTOSELECT, AN ANTHOCYANIN-RICH BILBERRY EXTRACT, ATTENUATES NON-ALCOHOLIC STEATOHEPATITIS AND ASSOCIATED FIBROSIS IN APOE*3LEIDEN MICE

Martine Morrison
Wen Liang
Petra Mulder
Lars Verschuren
Elsbet Pieterman
Karin Toet
Peter Heeringa
Peter Y. Wielinga
Teake Kooistra
Robert Kleemann

J Hepatol. 2014; S0168-8278(14): 00929-5

ABSTRACT

Background & Aims: Anthocyanins may have beneficial effects on lipid metabolism and inflammation and are demonstrated to have hepatoprotective properties in models of restraint-stress- and chemically-induced liver damage. However, their potential to protect against non-alcoholic steatohepatitis (NASH) under conditions relevant for human pathogenesis remains unclear. Therefore, we studied the effects of the standardized anthocyanin-rich extract Mirtoselect on diet-induced NASH in a translational model of disease.

Methods: ApoE*3Leiden mice were fed a Western-type cholesterol-containing diet without (HC) or with 0.1% (w/w) Mirtoselect (HCM) for 20 weeks to study effects on diet-induced NASH.

Results: Mirtoselect attenuated HC-induced hepatic steatosis, as observed by decreased macro- and microvesicular hepatocellular lipid accumulation and reduced hepatic cholesteryl-ester content. This anti-steatotic effect was accompanied by local anti-inflammatory effects in liver, as demonstrated by reduced inflammatory cell clusters and reduced neutrophil infiltration in HCM. On a molecular level, HC-diet significantly induced hepatic expression of pro-inflammatory genes Tnf, Emr1, Ccl2, Mpo, Cxcl1 and Cxcl2 while this induction was less pronounced or significantly decreased in HCM. A similar quenching effect was observed for HC-induced pro-fibrotic genes, Acta2 and Col1a1 and this anti-fibrotic effect of Mirtoselect was confirmed histologically. Many of the pro-inflammatory and pro-fibrotic parameters positively correlated with intrahepatic free cholesterol levels. Mirtoselect significantly reduced accumulation and crystallisation of intrahepatic free cholesterol, providing a possible mechanism for the observed hepatoprotective effects.

Conclusions: Mirtoselect attenuates development of NASH, reducing hepatic lipid accumulation, inflammation and fibrosis, possibly mediated by local anti-inflammatory effects associated with reduced accumulation and crystallisation of intrahepatic free cholesterol.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in Western countries [1, 2]. It constitutes a spectrum of liver injury, ranging from the clinically benign intrahepatic accumulation of lipids (steatosis), to the more progressive non-alcoholic steatohepatitis (NASH). In addition to hepatic lipid accumulation, NASH is characterised by hepatic inflammation, i.e. infiltration of immune cells [3] and can further progress to fibrosis, cirrhosis and hepatocellular carcinoma. Although the mechanisms by which NASH progresses are not completely understood, it is thought that dysregulation of cholesterol homeostasis and subsequent accumulation of free (unesterified) cholesterol are linked to the pathogenesis of NASH in humans (reviewed in reference [4]). In line with this notion, emerging experimental evidence implicates free cholesterol as a potential trigger of inflammation [5] as well as a possible driving factor in the development of fibrosis [6, 7]. A recent study in experimental and human NASH revealed that intrahepatic accumulation of free cholesterol can lead to the formation of cholesterol crystals in hepatocyte lipid droplets, which may form an important trigger for the progression of simple steatosis to NASH [8].

The anthocyanins, a subclass of the polyphenols, comprise a large group of bioactive compounds that are considered to have many health-promoting effects [9], including cholesterol-lowering [10, 11] and anti-inflammatory effects [12] which may mediate potential hepatoprotective properties [13]. Here we studied the effects of the standardised anthocyanin-rich bilberry (*Vaccinium myrtillus L.*) extract Mirtoselect on the development of NASH. This extract has been demonstrated to reduce circulating makers of inflammation in humans [14, 15] and has beneficial effects in restraint-stress- [16, 17] and chemically-induced [18] models of liver damage. However, its hepatoprotective potential in diet-induced metabolic inflammation and liver disease is unclear. Therefore we studied effects of Mirtoselect on NASH in ApoE*3Leiden (E3L) mice, a translational model of disease [19]. These mice develop diet-induced dyslipidaemia and inflammation on a high-fat/high-cholesterol diet [20], and ultimately develop NASH with fibrosis. Earlier studies have shown that E3L mice are sensitive to nutritional [21] and pharmacological [19] interventions, and show human-like responses to hypolipidaemic compounds [19, 22].

Combined histological, biochemical, and gene expression analyses revealed that Mirtoselect reduces development of NASH, attenuating both steatosis and inflammation as well as the development of hepatic fibrosis. These effects were associated with a reduction in hepatic free cholesterol accumulation and cholesterol crystal formation.

MATERIALS AND METHODS

Animal experiments

Experiments were approved by an independent Animal Care and Use Committee and were in compliance with European Community specifications regarding the use of laboratory animals. ApoE*3Leiden mice (E3L) were used because they allow study of diets and nutrients on lipids (including cholesterol) and liver inflammation [19, 23, 24].

Female E3L mice were fed a Western-type diet (15% cocoa butter, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch and 6.2% cellulose; diet-T; AB-Diets, Woerden, the Netherlands), supplemented with 1% (w/w) cholesterol (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 20 weeks. The study included a 4-week run-in during which all mice received this diet, after which they were matched for plasma cholesterol and triglycerides into 3 experimental groups (n=15/group). Control animals (HC) continued to receive the Western-type diet for the remainder of the study, while Mirtoselect-treated animals (HCM) received the HC diet with addition of 0.1% (w/w) Mirtoselect (Indena S.A.S., Paris, France). This standardised bilberry (*Vaccinium myrtillus* L.) extract contains 36% anthocyanins. An ageing reference group (REF) received the same Western-type diet mentioned above, but without cholesterol supplementation. Food intake and body weight were monitored throughout the study. Every 4 weeks, blood samples were collected via tail vein bleeding after a 4h fast, for isolation of EDTA plasma. Animals were sacrificed by CO_2 asphyxiation after 16 weeks of dietary treatment to collect livers. The medial lobe was fixed in formalin and embedded in paraffin for histological analysis of NASH and the left lobe was snap frozen in liquid nitrogen and stored at -80°C for cryosectioning, liver lipid- and mRNA-expression analyses.

Histological, biochemical and hepatic gene expression analyses

A detailed description of (immuno)histological, biochemical, and gene expression analyses is provided in (supplement 1). Briefly, development of NASH was assessed histologically using an adapted grading method for human NASH [25, 26]. Plasma lipids were determined with commercially available enzymatic assays and liver lipids were analysed by HPTLC, as described previously [27]. Hepatic gene expression analyses were performed by RT-PCR, using TaqMan® Gene Expression Assays (Life Technologies, Bleiswijk, the Netherlands) and changes in gene expression were calculated using the comparative Ct (ΔΔCt) method, expressed as fold-change relative to REF. Illumina microarray analysis of hepatic gene expression was performed following established normalisation and quality control protocols followed by gene enrichment analysis across pathways and biological processes as described [23]. p65-NFκB activity was determined in liver homogenates by DNA-binding ELISA (TransAM® p65-NFκB Chemi Kit, Active Motif, La Hulpe, Belgium) according to manufacturer's instructions and as described [26].

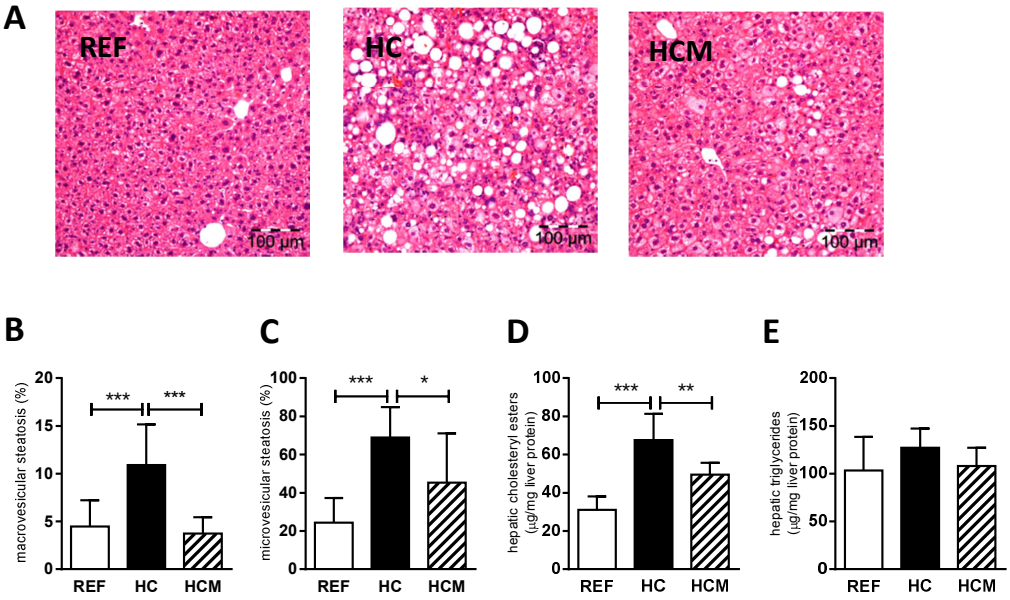
Statistical analyses

All data are presented as mean ± SD. Statistical analyses were performed using SPSS software (version 22, IBM, Armonk, USA). For normally distributed variables, significance of differences between groups was tested by one-way ANOVA, with Dunnett's Multiple Comparison Post-Hoc Test to compare HC vs REF and HC vs HCM. In case of heterogeneity between groups, variables were analysed by ANOVA using Brown-Forsythe for differences between groups with Dunnett's T3 Post-Hoc Test. Non-normally distributed variables were tested by non-parametric Kruskall-Wallis test followed by Mann-Whitney U. A p-value<0.05 was considered statistically significant.

RESULTS

Mirtoselect attenuates hepatic steatosis and hepatocellular damage

Treatments were well tolerated and there was no effect of Mirtoselect on food intake or body weight (supplement 2a-b). HC-feeding induced hepatosteatosis relative to REF, as observed histologically by a non-zonal accumulation of lipid macrovesicles and microvesicles in hepatocytes (**Figure 1a**). Mirtoselect attenuated the development of hepatic steatosis (**Figure 1a**), completely preventing the HC-induced increase in macrovesicular steatosis ($p<0.001$, **Figure 1b**) and strongly decreasing microvesicular steatosis ($p=0.027$, figure 1c). Analysis of intrahepatic lipid composition revealed that this increase in hepatic steatosis in HC was mainly attributable to an accumulation of lipids esterified to cholesterol (cholesteryl esters), the concentration of which was significantly lower in HCM ($p=0.008$, **Figure 1d**). Comparably, hepatic triglyceride levels (i.e. lipids esterified to glycerol) tended to build up in HC, but no increase was observed in HCM (**Figure 1e**). In association with steatosis, HC-feeding resulted in a pronounced induction of hepatocellular hypertrophy, the development of which was markedly reduced in HCM ($p=0.034$, supplement 3). In HC-fed animals, some of these hypertrophic cells were deficient in cytokeratin 18 (CK-18) compared with neighbouring cells (**Figure 1f**), indicating loss of cytoskeletal function and hepatocellular damage as seen in ballooning cells in human NASH [28, 29]. In Mirtoselect-treated mice, only very few enlarged CK-18 deficient cells were observed (**Figure 1f**), indicating a reduction in hepatocellular damage.



F

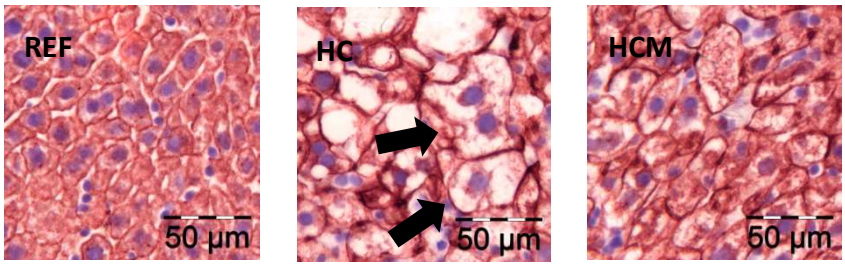


Figure 1: Mirtoselect attenuates hepatic steatosis in cholesterol-fed E3L-mice. Representative photomicrographs of liver sections of reference, high-cholesterol control and Mirtoselect-treated mice (A). Mirtoselect attenuated HC-diet-induced macrovesicular (B) and microvesicular (C) steatosis. Hepatic steatosis in HC-mice was mainly attributable to accumulation of cholesteryl-esters, the build-up of which was decreased by Mirtoselect (D). Hepatic triglycerides tended to accumulate in HC, which was not observed in HCM (E). In HC-fed animals, some hypertrophic cells were CK18-deficient compared with neighbouring cells (arrows); presence of these cells was reduced in HCM (F). REF: non-cholesterol-fed reference, HC: high-cholesterol control, HCM: high-cholesterol+Mirtoselect. Data are mean ± SD. *p<0.05, **p<0.01, ***p<0.001 compared with HC.

Mirtoselect reduces hepatic inflammation

In addition to hepatosteatosis, a defining characteristic of NASH is the presence of hepatic inflammation, which can be observed histologically as the lobular infiltration of inflammatory cells, i.e. inflammatory aggregates containing mononuclear cells (F4/80-positive cells of the monocyte/macrophage lineage) and polymorph nuclear cells (MPO-positive granulocytes, i.e. neutrophils). In comparison with REF, the number of inflammatory cell aggregates increased strongly in HC, and this HC-induced hepatic inflammatory response was fully blunted with Mirtoselect (p<0.001, **Figure 2a**). Investigation of hepatic *Emr1* (F4/80) gene expression revealed that the influx of inflammatory cells was partly attributable to macrophages, which was supported by increased gene expression levels of *Ccl2* (MCP-1), a mediator of monocyte recruitment (**Figure 2b-c**). Mirtoselect did not affect expression of *Emr1*or *Ccl2* (**Figure 2b-c**), indicating that its anti-inflammatory effect may impair the influx of another immune cell type. Hepatic gene expression analysis of the neutrophil marker *Mpo* showed that *Mpo* expression was increased in HC animals and this induction was completely prevented in HCM animals (p=0.034, **Figure 2d**). Immunohistochemical staining of MPO-positive cells confirmed the mRNA expression data and showed that HC induced neutrophil infiltration, which was attenuated by HCM (**Figure 2e**). In line with these findings, the expression of two neutrophil chemoattractants – *Cxcl1* and *Cxcl2* – was upregulated strongly and significantly by HC feeding while induction of these chemokines was less pronounced in HCM animals (*Cxcl1*: p=0.327, *Cxcl2*: p=0.131, **Figure 2f-g**).

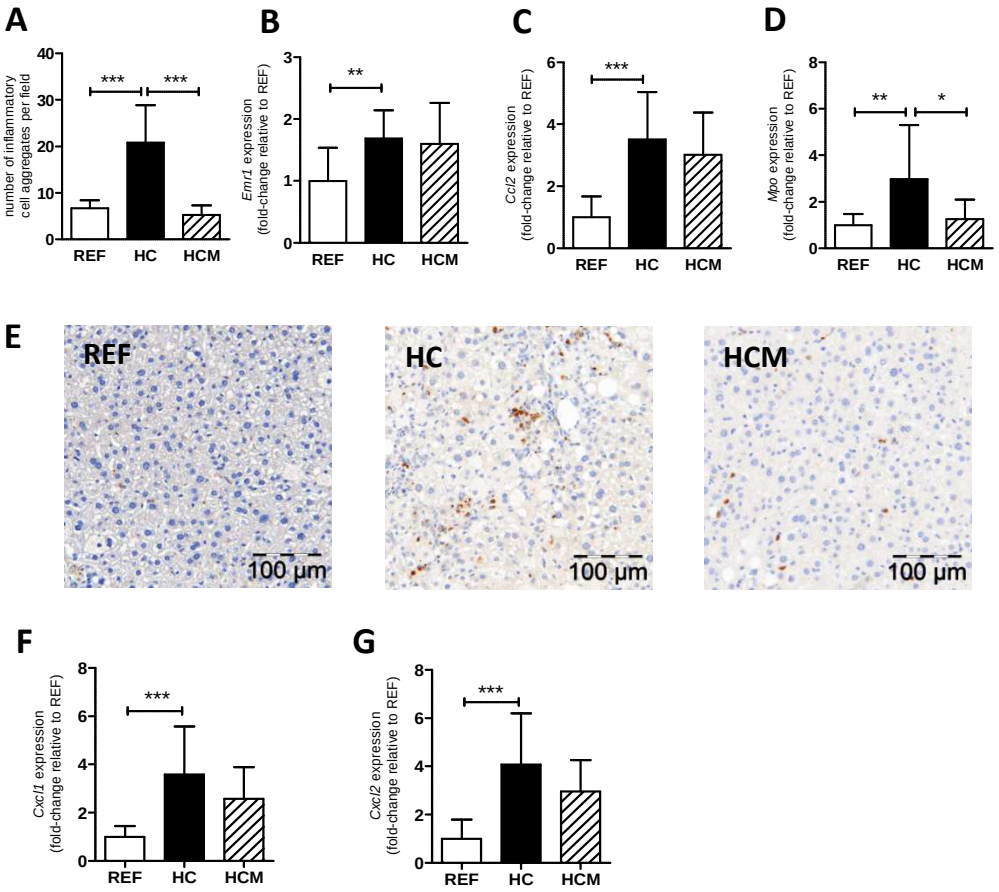


Figure 2: Mirtoselect reduces hepatic inflammation in cholesterol-fed E3L-mice. Number of HC-induced inflammatory cell aggregates was reduced by Mirtoselect (A). HC-diet induced Emr1 (B) and Ccl2 (C) gene expression was not affected by Mirtoselect. Mirtoselect reduced HC-induced Mpo gene expression (D) and number of MPO-positive cells as determined immunohistochemically (E). HC-induced gene expression of neutrophil chemoattractants Cxcl1 (F) and Cxcl2 (G) was less pronounced in HCM. REF: non-cholesterol-fed reference, HC: high-cholesterol control, HCM: high-cholesterol+Mirtoselect. Data are mean ± SD. *p<0.05, **p<0.01, ***p<0.001 compared with HC.

Mirtoselect attenuates hepatic fibrosis

Continued hepatic inflammation is thought to drive the progression of NASH, ultimately resulting in the development of hepatic fibrosis. Histochemical staining of hepatic collagen content by Picro-Sirius Red staining demonstrated that the HC diet caused liver fibrosis,

characterised by periportal, pericentral and perisinusoidal deposition of collagen. This HC-induced fibrosis was much less pronounced in HCM (**Figure 3a**).

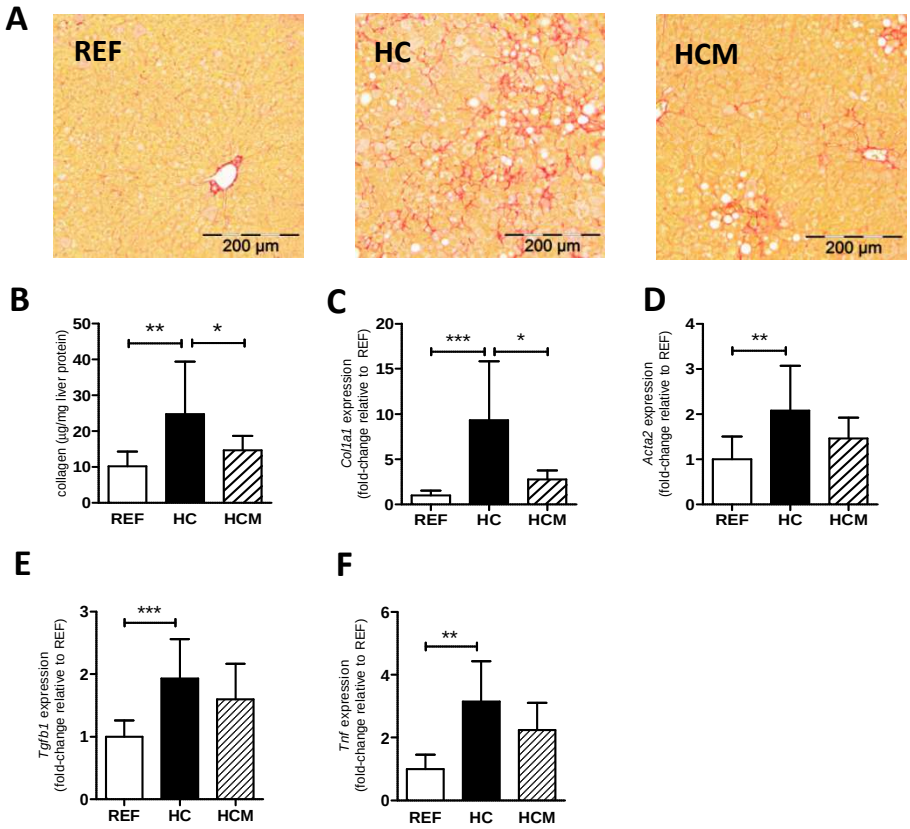


Figure 3: Development of hepatic fibrosis in cholesterol-fed E3L-mice reduced by Mirtoselect. Representative photomicrographs of picro-sirius red-stained liver sections show reduction of HC-induced collagen content in Mirtoselect-treated mice (A), which was confirmed by biochemical analysis of hepatic collagen content (B). Induction of Col1a1 gene expression is prevented by Mirtoselect (C). Gene expression of hepatic stellate cell activation marker Acta2 (D) as well as pro-fibrotic cytokines Tgfb1 (E) and Tnf (F) was induced by HC, while this induction was less pronounced in HCM. REF: non-cholesterol-fed reference, HC: high-cholesterol control, HCM: high-cholesterol+Mirtoselect. Data are mean ± SD. *p<0.05, **p<0.01, ***p<0.001 compared with HC.

In line with these histological observations, biochemical analysis of hepatic collagen content revealed a pronounced increase in collagen content in HC compared with REF, which was significantly reduced in HCM (p=0.034, **Figure 3b**) and hepatic gene expression analysis of *Col1a1* showed significantly increased expression in HC compared with REF, while Mirtoselect quenched this effect and significantly reduced *Col1a1* expression compared with HC (p=0.011, **Figure 3c**). Additionally, HC diet significantly induced expression of the hepatic stellate cell activation marker *Acta2* (α-SMA) (**Figure 3d**), as well

as the pro-fibrotic cytokines *Tgfb1* (TGF-β) and *Tnf* (TNF-α) (**Figure 3e-f**). In line with the attenuating effect of Mirtoselect on hepatic fibrosis development, the induction of these genes was less pronounced, although non-significantly, in HCM (*Acta2:* p=0.173, *Tgfb1:* p=0.174, *Tnf:* p=0.096, **Figure 3d-f**). Subsequent microarray pathway analysis revealed that many genes downstream of TGF-β were affected, indicating strongly and significantly activated TGF-β signalling in HC compared with REF (z=7.539, p=2.5E-51). Mirtoselect strongly inhibited activation of this pathway (z=-4.862, p=3.3E-20 compared with HC). Consistent with this, the process 'Hepatic fibrosis/hepatic stellate cell activation', was strongly activated in HC compared with REF and Mirtoselect suppressed this activation (supplement 4). Together, our data demonstrate that HC-feeding induced histopathological and molecular hallmarks of NASH and fibrosis and that Mirtoselect significantly attenuated disease development.

Intrahepatic free cholesterol correlates with development of NASH and is reduced by Mirtoselect

To gain more insight into the metabolic-inflammatory processes that drive the development of NASH and hepatic fibrosis and the effects of Mirtoselect thereupon, we next analysed a possible metabolic trigger of inflammation: cholesterol. HC-feeding induced dyslipidaemia with increased plasma total cholesterol compared with REF, specifically in the VLDL- and LDL-sized particles. Mirtoselect did not affect circulating cholesterol levels or lipoprotein profile (supplement 5) pointing to a hepatoprotective effect within the liver tissue. As intrahepatic free cholesterol is a very potent inducer of liver inflammation [23] and is elevated intrahepatically in human NASH [30, 31], we determined free cholesterol concentrations in freshly prepared liver homogenates and correlated them with histology scores and gene expression data. Both hepatic inflammation (number of inflammatory clusters observed histologically, p<0.001), and hepatic fibrosis (hepatic *Col1a1* expression, p=0.007) were positively correlated with hepatic free cholesterol levels (**Figure 4a-b**). Further strengthening this notion, we also observed positive significant correlations of hepatic free cholesterol levels with the expression level of many of the investigated pro-inflammatory and pro-fibrotic parameters (i.e. *Ccl2, Cxcl1, Cxcl2, Acta2, Tgfb1* and *Tnf,* shown in supplement 6). Importantly, Mirtoselect fully blunted the disease-associated increase in hepatic free cholesterol, the concentrations of which were comparable to REF and significantly lower than in HC (p=0.008, **Figure 4c**). To further examine the link between cholesterol and inflammation and the effects of Mirtoselect thereupon we analysed activation of inflammatory pathways by microarray as well as biochemically. HC diet significantly induced TNF-α and IL-1β signalling (TNF-α: z=7.539, p=2.5E-51; IL-1β: z=6.516, p=4.70E-30; vs REF) and activated the downstream pro-inflammatory transcription factor NFκB (z=6.245, p=4.82E-14 vs REF). Consistent with this, hepatic free cholesterol levels were positively correlated with biochemically measured transcriptional activation of p65-NFκB (R^2=0.51, p=0.021), providing a link between cholesterol and inflammation. Mirtoselect significantly reduced p65-NFκB activity relative to HC (fold-change relative to REF: 1.15 ± 0.11 in HC vs 0.98 ± 0.05 in HCM, p=0.032).

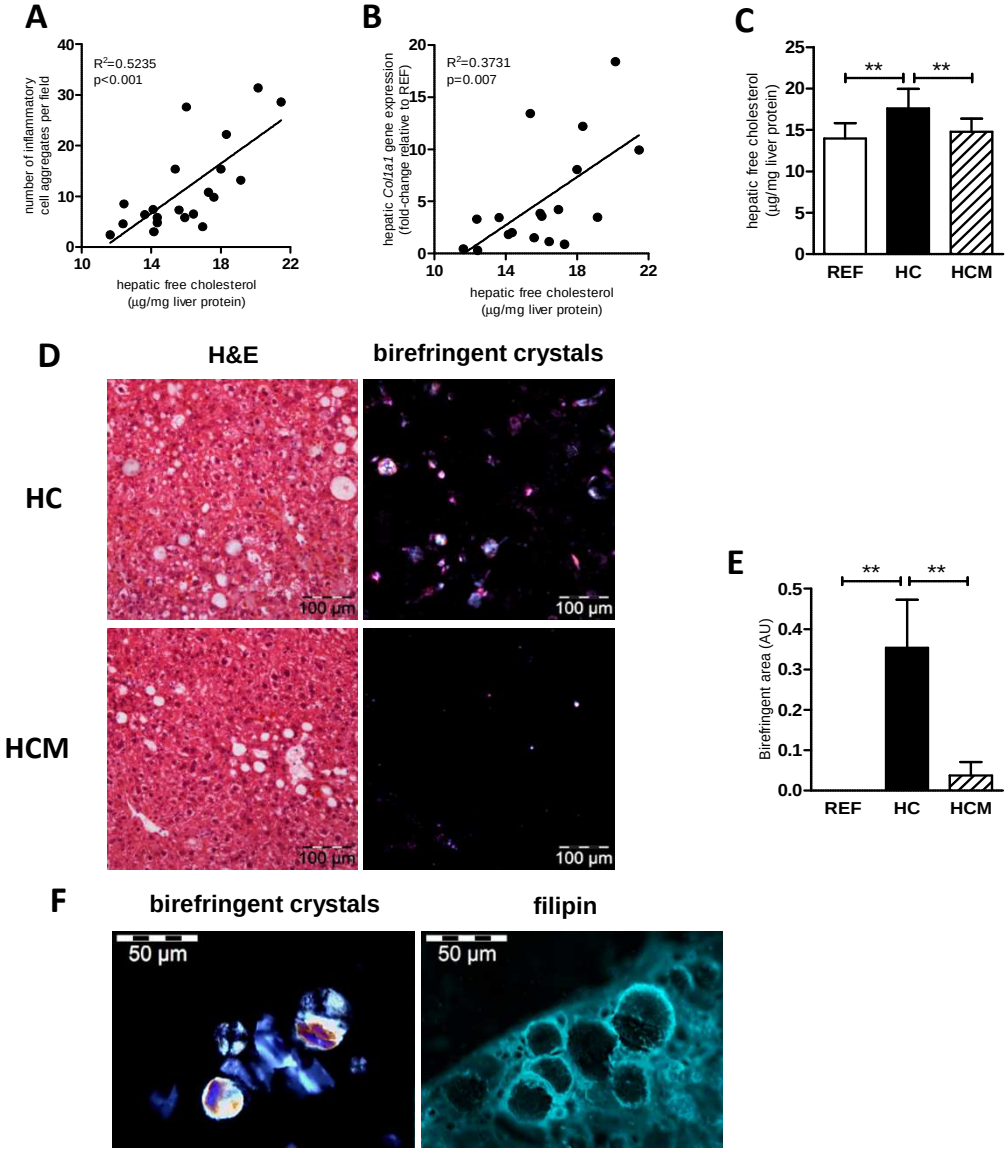


Figure 4: Mirtoselect reduces intrahepatic free cholesterol accumulation and crystallisation in cholesterol-fed E3L-mice. Intrahepatic free cholesterol levels correlated with hepatic inflammation (inflammatory aggregates per field; A) and hepatic fibrosis (Col1a1 expression; B). HC-induced hepatic free cholesterol accumulation was not observed in HCM (C). Representative photomicrographs of liver cryosections (same field under bright-field and polarised-light microscopy) reveal presence of birefringent crystals in HC that were hardly observed in HCM (D). Quantification of birefringent area shows strong HC-induced increase that is not observed in HCM (E). Birefringent crystals stained prominently with free-cholesterol staining filipin (same field under polarised-light and fluorescent microscopy; F). REF: non-cholesterol-fed reference, HC: high-cholesterol control, HCM: high-cholesterol+Mirtoselect. Data are mean ± SD. **p<0.01 compared with HC.

More detailed analysis of processes that may underlie observed effects of Mirtoselect on hepatic cholesterol accumulation showed that plasma markers of cholesterol uptake (plant sterols to cholesterol ratio and cholestanol to cholesterol ratio) or cholesterol biosynthesis (lathosterol to cholesterol ratio) were not affected in HCM (supplement 7). Also, microarray analysis confirmed absence of an effect of Mirtoselect on cholesterol biosynthesis but revealed a significant activation of FXR in HCM (z=2.190, p=7.64E-03) and showed that genes involved in bile acid synthesis, bile acid conjugation and bile salt secretion (e.g. Bsep, Cyp27a1) were upregulated in HCM.

Refined microscopic analysis of liver cross-sections under polarised light revealed that HC-feeding caused pronounced formation of large birefringent crystals within a considerable amount of the macrovesicular lipid droplets, while these crystals were hardly observed in Mirtoselect-treated animals (even in the few regions containing macrovesicles, **Figure 4d-e**). These birefringent crystals stained positively with filipin (**Figure 4f**), which forms a fluorescent complex with free cholesterol specifically, indicating that the observed birefringence is attributable to crystallised free cholesterol. Detailed examination of the liver cross-sections under bright field and polarised light microscopy revealed that many of the hepatocytes containing these crystals were devoid of normal cellular morphology and organisation. The prevention of this intrahepatic cholesterol crystal formation by Mirtoselect provides a possible rationale for its observed hepatoprotective properties.

DISCUSSION

We investigated the potential hepatoprotective properties of a standardised anthocyanin-rich extract (Mirtoselect) in a diet-induced, translational model of NASH with fibrosis. We show that Mirtoselect protects against the development of NASH, reducing hepatic steatosis, hepatic inflammation and hepatic fibrosis, associated with decreased accumulation and crystallisation of intrahepatic free cholesterol. The observed hepatoprotective effects of Mirtoselect were achieved at a dosage that translates to an anthocyanin intake of around 300 mg/day in humans, an intake that is achievable by diet [12, 32, 33].

HC diet-feeding induced intrahepatic lipid accumulation characterised by macro- and microvesicular steatosis, which was mainly attributable to an increase in lipids esterified to cholesterol. Mirtoselect strongly reduced this intrahepatic accumulation of lipids. Experimental support for these observed anti-steatotic effects is provided by results from earlier studies that report improved hepatic cholesterol homeostasis in rodents treated with similar concentrations of anthocyanin-rich extracts [11, 34] or individual anthocyanins [35]. Although these studies were not performed under conditions leading to NAFLD/NASH, they do provide a possible mechanism for the observed reduction in intrahepatic cholesterol: intervention with anthocyanins was found to increase bile acid synthesis [34, 35] and faecal sterol excretion [11, 35], thereby decreasing intrahepatic cholesterol accumulation [11, 35] which is consistent with our observations of FXR activation by Mirtoselect. Although it is the anthocyanin fraction of Mirtoselect that is considered to be the principal bioactive fraction of the extract, it is unknown which of

these anthocyanins or other possibly bioactive constituents (alone or in combination), may be responsible for the observed effects.

In addition to reducing hepatic steatosis Mirtoselect also attenuated hepatic inflammation, completely preventing the HC-induced increase in inflammatory cell aggregates. This anti-inflammatory effect was largely attributable to an effect specifically on the influx of MPO-positive neutrophils, the infiltration of which is recognised as a defining characteristic of inflammation in human NASH [36]. The exact role of neutrophils in the pathogenesis of NASH remains to be elucidated [37], but their ability to release a potent cocktail of reactive oxygen species and proteases implicates them as potential cause of extensive tissue damage [38] that may contribute to amplification of the inflammatory response as well as development of fibrosis. We observed a marked reduction of neutrophils in livers of Mirtoselect-treated mice, but only a modest effect on the hepatic expression of neutrophil chemoattractants *Cxcl1* and *Cxcl2,* suggesting additional mechanisms outside the liver. Indeed, it has been demonstrated that anthocyanins can attenuate the induction of chemokine receptors such as CXCR2 [39], which is required for neutrophil chemotaxis.

Although inflammation is recognised to play an important role in the development of NASH, the nature of the trigger for this inflammatory component remains unclear. Lipotoxicity caused by the build-up of toxic lipid species is thought to play an important role, but the specific lipid species that mediate hepatic lipotoxicity have not been identified with certainty [40]. While triglycerides are the main accumulating lipid species in human NASH, recent studies have implicated free cholesterol as a potential trigger for disease progression [4]. The experimental conditions chosen for the present study emphasize the role of cholesterol in NASH, and limit the study of hepatic triglyceride accumulation. Results from epidemiological studies that link dietary cholesterol intake to increased risk and severity of NAFLD [41, 42] and cirrhosis [43] provide indications that cholesterol may play a causal role in NASH development. In support of this notion, free cholesterol is increased intrahepatically in human [30, 31] and experimental NASH [44] and modulation of hepatic free cholesterol levels by diet [44] or pharmacological intervention [45] is closely linked to the severity of experimental NASH. Furthermore, there are indications that cholesterol-lowering agents (e.g. statins, ezetimibe) may improve NASH in patients with hypercholesterolaemia [46, 47]. Mechanistic studies have shown that free cholesterol accumulation in Kupffer cells [48] and hepatic stellate cells (HSC) [6, 7] promotes inflammation and exacerbates fibrosis (e.g. increased TNF-α and CCL2 expression by Kupffer cells and increased COL1A1 expression in HSC). Results from the study described herein show that intrahepatic free cholesterol levels are positively correlated with many factors that contribute to or reflect progressive development of NASH (e.g. *Ccl2, Cxcl1, Cxcl2, Col1a1, Acta2, Tnf,* and *Tgfb1*). The observed activation of inflammatory signalling routes (IL-1β, TNF-α, TGF-β) by HC as well as the positive correlation between free cholesterol and NFκB activity, point to NFκB activation as an effector of cholesterol-induced inflammation, which is in line with previous observations [23, 27]. Mirtoselect decreases the build-up of this cytotoxic lipid species and associated NFκB activation, thereby providing a possible explanation for its beneficial effects on NAFLD development.

A recently emerging mechanism of cellular toxicity associated with free cholesterol accumulation is intracellular cholesterol crystallisation, which can happen when the concentration of free cholesterol reaches a very high level [49]. Cholesterol crystals, particularly when they are very small (nm range), can trigger inflammation through inflammasome activation [5, 50]. Besides putative pro-inflammatory effects of cholesterol crystals [5, 50], it is plausible that the sheer size of the intrahepatocellular cholesterol crystals observed in the present study (with diameters ranging up to 50 µm) would also cause extensive physical damage to the cells containing them. Indeed, the formed cholesterol crystals may damage cells by physically disrupting the integrity of intracellular structures [49], in line with observations in the present study. Furthermore, cellular damage can result in the release of damage associated molecular patterns (DAMPs), which leads to neutrophil recruitment [37] further enhancing the inflammatory response. Additional support for this mechanism is provided by results from human studies that demonstrate that cholesterol crystals distinguish NASH from simple steatosis [8] and that this accumulation and crystallisation of free cholesterol within steatotic hepatocytes may be an important trigger for disease progression.

Overall, we show that Mirtoselect has beneficial effects in NASH, improving hepatic steatosis, inflammation and fibrosis. Furthermore, we demonstrate the presence of cholesterol crystals and associated tissue damage in NASH and show that dietary intervention with Mirtoselect prevents this accumulation and crystallisation of free cholesterol, providing a possible rationale for its hepatoprotective effects. Given the moderate dose of Mirtoselect used, this study suggests that intervention with naturally occurring, well-tolerated polyphenols may constitute a powerful approach to retard NASH development.

ACKNOWLEDGEMENTS

We would like to thank Wim van Duyvenvoorde and Erik Offerman for their excellent technical assistance.

REFERENCES

[1] Browning J D, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology 2004; 40:1387-1395.

[2] Clark J M. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol 2006; 40 Suppl 1:S5-10.

[3] Fujii H, Kawada N. Inflammation and fibrogenesis in steatohepatitis. J Gastroenterol 2012; 47:215-225.

[4] Musso G, Gambino R, Cassader M. Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis. Prog Lipid Res 2013; 52:175-191.

[5] Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 2010; 464:1357-1361.

[6] Teratani T, Tomita K, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K et al. A high-cholesterol diet exacerbates liver fibrosis in mice via

accumulation of free cholesterol in hepatic stellate cells. Gastroenterology 2012; 142:152-164.e10.
[7] Tomita K, Teratani T, Suzuki T, Shimizu M, Sato H, Narimatsu K et al. Free cholesterol accumulation in hepatic stellate cells: mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice. Hepatology 2014; 59:154-169.
[8] Ioannou G N, Haigh WG, Thorning D, Savard C. Hepatic cholesterol crystals and crown-like structures distinguish NASH from simple steatosis. J Lipid Res 2013; 54:1326-1334.
[9] He J, Giusti MM. Anthocyanins: natural colorants with health-promoting properties. Annu Rev Food Sci Technol 2010; 1:163-187.
[10] Qin Y, Xia M, Ma J, Hao Y, Liu J, Mou H et al. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. Am J Clin Nutr 2009; 90:485-492.
[11] Liang Y, Chen J, Zuo Y, Ma KY, Jiang Y, Huang Y et al. Blueberry anthocyanins at doses of 0.5 and 1 % lowered plasma cholesterol by increasing fecal excretion of acidic and neutral sterols in hamsters fed a cholesterol-enriched diet. Eur J Nutr 2013; 52:869-875.
[12] Jennings A, Welch AA, Spector T, Macgregor A, Cassidy A. Intakes of anthocyanins and flavones are associated with biomarkers of insulin resistance and inflammation in women. J Nutr 2014; 144:202-208.
[13] Valenti L, Riso P, Mazzocchi A, Porrini M, Fargion S, Agostoni C. Dietary anthocyanins as nutritional therapy for nonalcoholic fatty liver disease. Oxid Med Cell Longev 2013; 2013:145421.
[14] Karlsen A, Paur I, Bohn SK, Sakhi AK, Borge GI, Serafini M et al. Bilberry juice modulates plasma concentration of NF-kappaB related inflammatory markers in subjects at increased risk of CVD. Eur J Nutr 2010; 49:345-355.
[15] Kolehmainen M, Mykkanen O, Kirjavainen PV, Leppanen T, Moilanen E, Adriaens M et al. Bilberries reduce low-grade inflammation in individuals with features of metabolic syndrome. Mol Nutr Food Res 2012; 56:1501-1510.
[16] Bao L, Yao XS, Yau CC, Tsi D, Chia CS, Nagai H et al. Protective effects of bilberry (Vaccinium myrtillus L.) extract on restraint stress-induced liver damage in mice. J Agric Food Chem 2008; 56:7803-7807.
[17] Bao L, Abe K, Tsang P, Xu JK, Yao XS, Liu HW et al. Bilberry extract protect restraint stress-induced liver damage through attenuating mitochondrial dysfunction. Fitoterapia 2010; 81:1094-1101.
[18] Domitrovic R, Jakovac H. Effects of standardized bilberry fruit extract (Mirtoselect(R)) on resolution of CCl4-induced liver fibrosis in mice. Food Chem Toxicol 2011; 49:848-854.
[19] Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM et al. Mouse models for atherosclerosis and pharmaceutical modifiers. Arterioscler Thromb Vasc Biol 2007; 27:1706-1721.
[20] Duivenvoorden I, Voshol PJ, Rensen PC, van Duyvenvoorde W, Romijn JA, Emeis JJ et al. Dietary sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver steatosis in APOE*3Leiden mice. Am J Clin Nutr 2006; 84:312-321.
[21] Morrison M, van der Heijden R, Heeringa P, Kaijzel E, Verschuren L, Blomhoff R et al. Epicatechin attenuates atherosclerosis and exerts anti-inflammatory effects on diet-induced human-CRP and NFkappaB in vivo. Atherosclerosis 2014; 233:149-156.
[22] van de Steeg E, Kleemann R, Jansen HT, van Duyvenvoorde W, Offerman EH, Wortelboer HM et al. Combined analysis of pharmacokinetic and efficacy data of preclinical studies with statins markedly improves translation of drug efficacy to human trials. J Pharmacol Exp Ther 2013; 347:635-644.
[23] Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER et al. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. Genome Biol 2007; 8:R200.

[24] Verschuren L, Wielinga PY, van Duyvenvoorde W, Tijani S, Toet K, van Ommen B et al. A dietary mixture containing fish oil, resveratrol, lycopene, catechins, and vitamins E and C reduces atherosclerosis in transgenic mice. J Nutr 2011; 141:863-869.

[25] Kleiner D E, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005; 41:1313-1321.

[26] Liang W, Lindeman JH, Menke AL, Koonen DP, Morrison M, Havekes LM et al. Metabolically induced liver inflammation leads to NASH and differs from LPS- or IL-1beta-induced chronic inflammation. Lab Invest 2014; 94:491-502.

[27] Wielinga P Y, Yakala GK, Heeringa P, Kleemann R, Kooistra T. Beneficial effects of alternate dietary regimen on liver inflammation, atherosclerosis and renal activation. PLoS One 2011; 6:e18432.

[28] Caldwell S, Ikura Y, Dias D, Isomoto K, Yabu A, Moskaluk C et al. Hepatocellular ballooning in NASH. J Hepatol 2010; 53:719-723.

[29] Zatloukal K, Stumptner C, Fuchsbichler A, Fickert P, Lackner C, Trauner M et al. The keratin cytoskeleton in liver diseases. J Pathol 2004; 204:367-376.

[30] Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O et al. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology 2007; 46:1081-1090.

[31] Caballero F, Fernandez A, De Lacy AM, Fernandez-Checa JC, Caballeria J, Garcia-Ruiz C. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. J Hepatol 2009; 50:789-796.

[32] Hertog M G, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 1993; 20:21-29.

[33] Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB. High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. Circulation 2013; 127:188-196.

[34] Mauray A, Felgines C, Morand C, Mazur A, Scalbert A, Milenkovic D. Bilberry anthocyanin-rich extract alters expression of genes related to atherosclerosis development in aorta of apo E-deficient mice. Nutr Metab Cardiovasc Dis 2012; 22:72-80.

[35] Wang D, Xia M, Gao S, Li D, Zhang Y, Jin T et al. Cyanidin-3-O-beta-glucoside upregulates hepatic cholesterol 7alpha-hydroxylase expression and reduces hypercholesterolemia in mice. Mol Nutr Food Res 2012; 56:610-621.

[36] Brunt E M, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999; 94:2467-2474.

[37] Xu R, Huang H, Zhang Z, Wang FS. The role of neutrophils in the development of liver diseases. Cell Mol Immunol 2014; 11:224-231.

[38] Kubes P, Mehal WZ. Sterile inflammation in the liver. Gastroenterology 2012; 143:1158-1172.

[39] Kang M K, Li J, Kim JL, Gong JH, Kwak SN, Park JH et al. Purple corn anthocyanins inhibit diabetes-associated glomerular monocyte activation and macrophage infiltration. Am J Physiol Renal Physiol 2012; 303:F1060-9.

[40] Farrell G C, van Rooyen D, Gan L, Chitturi S. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. Gut Liver 2012; 6:149-171.

[41] Musso G, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M et al. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology 2003; 37:909-916.

[42] Yasutake K, Nakamuta M, Shima Y, Ohyama A, Masuda K, Haruta N et al. Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: the significance of dietary cholesterol. Scand J Gastroenterol 2009; 44:471-477.

[43] Ioannou G N, Morrow OB, Connole ML, Lee SP. Association between dietary nutrient composition and the incidence of cirrhosis or liver cancer in the United States population. Hepatology 2009; 50:175-184.

[44] Van Rooyen D M, Larter CZ, Haigh WG, Yeh MM, Ioannou G, Kuver R et al. Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. Gastroenterology 2011; 141:1393-403, 1403.e1-5.

[45] Van Rooyen D M, Gan LT, Yeh MM, Haigh WG, Larter CZ, Ioannou G et al. Pharmacological cholesterol lowering reverses fibrotic NASH in obese, diabetic mice with metabolic syndrome. J Hepatol 2013; 59:144-152.

[46] Ekstedt M, Franzen LE, Mathiesen UL, Holmqvist M, Bodemar G, Kechagias S. Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: a histopathological follow-up study. J Hepatol 2007; 47:135-141.

[47] Yoneda M, Fujita K, Nozaki Y, Endo H, Takahashi H, Hosono K et al. Efficacy of ezetimibe for the treatment of non-alcoholic steatohepatitis: An open-label, pilot study. Hepatol Res 2010; 40:566-573.

[48] Leroux A, Ferrere G, Godie V, Cailleux F, Renoud ML, Gaudin F et al. Toxic lipids stored by Kupffer cells correlates with their pro-inflammatory phenotype at an early stage of steatohepatitis. J Hepatol 2012; 57:141-149.

[49] Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. J Clin Invest 2002; 110:905-911.

[50] Samstad E O, Niyonzima N, Nymo S, Aune MH, Ryan L, Bakke SS et al. Cholesterol Crystals Induce Complement-Dependent Inflammasome Activation and Cytokine Release. J Immunol 2014; 192:2837-45.