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RESEARCH PAPER

# Haloperidol inhibits the development of atherosclerotic lesions in LDL receptor knockout mice

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## BACKGROUND AND PURPOSE

Antipsychotic drugs have been shown to modulate the expression of ATP-binding cassette transporter A1 (ABCA1), a key factor in the anti-atherogenic reverse cholesterol transport process, *in vitro*. Here we evaluated the potential of the typical antipsychotic drug haloperidol to modulate the cholesterol efflux function of macrophages *in vitro* and their susceptibility to atherosclerosis *in vivo*.

## EXPERIMENTAL APPROACH

Thioglycollate-elicited peritoneal macrophages were used for *in vitro* studies. Hyperlipidaemic low-density lipoprotein (LDL) receptor knockout mice were implanted with a haloperidol-containing pellet and subsequently fed a Western-type diet for 5 weeks to induce the development of atherosclerotic lesions *in vivo*.

## KEY RESULTS

Haloperidol induced a 54% decrease in the mRNA expression of ABCA1 in peritoneal macrophages. This coincided with a 30% decrease in the capacity of macrophages to efflux cholesterol to apolipoprotein A1. Haloperidol treatment stimulated the expression of ABCA1 (+51%) and other genes involved in reverse cholesterol transport, that is, CYP7A1 (+98%) in livers of LDL receptor knockout mice. No change in splenic ABCA1 expression was noted. However, the average size of the atherosclerotic size was significantly smaller (–31%) in the context of a mildly more atherogenic metabolic phenotype upon haloperidol treatment. More importantly, haloperidol markedly lowered MCP-1 expression (–70%) and secretion (–28%) by peritoneal macrophages.

## CONCLUSIONS AND IMPLICATIONS

Haloperidol treatment lowered the susceptibility of hyperlipidaemic LDL receptor knockout mice to develop atherosclerotic lesions. Our findings suggest that the beneficial effect of haloperidol on atherosclerosis susceptibility can be attributed to its ability to inhibit macrophage chemotaxis.

## Abbreviations

ABCA1, ATP-binding cassette transport A1; ABCG1, ATP-binding cassette transport G1; ABCG5, ATP-binding cassette transport G5; APOA1, apolipoprotein A1; CYP7A1, cholesterol 7α-hydroxylase; FASN, fatty acid synthase; Halo, haloperidol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LXR, liver X receptor; MCP-1 (CCL2), monocyte chemoattractant protein-1; OGTT, oral glucose tolerance test; QTc, corrected QT; SCD1, stearoyl-CoA desaturase 1; SR-BI, scavenger receptor BI; VLDL, very-low-density lipoprotein

## Tables of Links

| TARGETS | |
|---|---|
| **Nuclear hormone receptors**[a] | **Enzymes**[c] |
| LXR | CYP7A1 |
| **Transporters**[b] | Fatty acid synthase |
| ABCA1 | |
| ABCG1 | |
| ABCG5 | |

| LIGANDS | |
|---|---|
| Cholesterol | IL-10 |
| Clozapine | Imipramine |
| Haloperidol | Isoflurane |
| IFN-γ | MCP-1 (CCL2) |
| IL-4 | TNF-α |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

## Introduction

Atherosclerosis is the primary underlying cause of cardiovascular disease. High plasma concentrations of cholesterol associated with low-density lipoproteins (LDLs) are an established risk factor for the development of atherosclerosis, while a strong negative correlation exists between levels of cholesterol in the high-density lipoprotein (HDL) fraction and atherosclerosis burden (Castelli *et al.*, 1977). LDL-cholesterol lowering by statins represents the first line treatment of subjects at risk of cardiovascular disease. Statin treatment only reduces the cardiovascular risk by 25–30% (Cholesterol Treatment Trialists' (CTT) Collaborators *et al.*, 2012), which highlights the need for alternative treatment approaches.

Under hyperlipidaemic conditions, macrophages take up excess amounts of cholesterol leading to the formation of lipid-filled foam cells that lose their ability to migrate. The accumulation of macrophage foam cells locally within the arterial wall is a hallmark in the pathogenesis of atherosclerosis. Because macrophages are unable to limit the uptake of cholesterol, they rely on reverse cholesterol transport, that is, the efflux of cholesterol from cells for subsequent excretion via the liver, to maintain non-pathological intracellular cholesterol levels. We and others have identified the energy-dependent efflux of cholesterol by the full-size ATP-binding cassette transporter A1 (ABCA1) as a primary route of cholesterol disposal by macrophages and a key factor in the protection against atherosclerosis. More specifically, genetic disruption of ABCA1 function in macrophages is associated with an enhanced susceptibility for the development of foam cells and atherosclerotic lesions (Aiello *et al.*, 2002; Van Eck *et al.*, 2002), while overexpression of macrophage ABCA1 delays the initiation of atherosclerosis in hyperlipidaemic mice (Van Eck *et al.*, 2006).

Interestingly, *in vitro* studies by Vik-Mo *et al.* (2009) have suggested that psychotropic drugs such as haloperidol, clozapine and imipramine change the expression of ABCA1 in cultured cells. Here we evaluated the potential of the typical antipsychotic drug haloperidol to modulate the cholesterol efflux function of macrophages *in vitro* and their susceptibility to atherosclerosis *in vivo*.

## Methods

### *Mice*

Animal experiments were performed at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research. Female homozygous LDL receptor knockout mice, 13–16 weeks old, were bred in house at the Gorlaeus Laboratories. Food and water were supplied *ad libitum*. A total of 25 mice (12 control; 13 haloperidol) were used in the experiment. Mice were housed in groups of four to five in a temperature- and light-controlled environment. All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare, and the experimental protocol was approved by the Animal Ethics Committee of Leiden University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

### *Isolation of peritoneal cells and cell culture*

Mice were injected i.p. with 3% Brewer's thioglycollate medium to induce the infiltration of macrophages into the peritoneal cavity. At 5 days after injection, the peritoneal cavity of the mice was lavaged with 10 mL cold PBS to collect thioglycollate-elicited peritoneal leukocytes. Peritoneal macrophages were incubated in DMEM containing 10% FBS for 24 h in the presence or absence of haloperidol to determine its effect on gene expression and cytokine secretion.

### *Gene expression by real-time quantitative PCR*

Total RNA was extracted by the acid guanidinium thiocyanate-phenol chloroform extraction method according to Chomczynski and Sacchi (1987). RNA concentrations were measured using the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 1 μg of total RNA, and mRNA levels were quantitatively determined as described previously (Hoekstra *et al.*, 2003). PCR primers were designed using Primer Express 1.5 Software with the manufacturer's default settings (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are available upon

request. Expression levels were normalized to those of ribosomal protein S13, hypoxanthine-guanine phosphoribosyltransferase, β-actin and ribosomal protein 36B4.

### Cytokine measurements

Murine monocyte chemoattractant protein-1 (MCP-1) levels were assayed in cell culture medium using a MCP-1 instant ELISA kit (eBioscience, Hatfield, UK) according to the manufacturer's instructions.

### Cholesterol efflux assay

To measure the cholesterol efflux capacity of the cells, thioglycollate-elicited peritoneal macrophages were incubated with 0.5 μCi·mL$^{-1}$ [$^3$H]-cholesterol in DMEM/0.2% BSA (fatty acid free) for 24 h at 37°C. Cholesterol efflux was studied by incubation of the cells with DMEM/0.2% BSA alone or supplemented with either 10 μg·mL$^{-1}$ apoAI (Calbiochem, San Diego, CA, USA) or 50 μg·mL$^{-1}$ human HDL (density 1.063–1.21 g·mL$^{-1}$) as lipid acceptors in the presence or absence of 10 μM haloperidol. Radioactivity in the medium and the cells was determined by scintillation counting after 24 h of incubation. Cholesterol efflux was calculated as the amount of radioactivity in the medium compared with the total amount of radioactivity measured in medium plus cells.

### Atherosclerosis study design

Mice were randomly assigned to either haloperidol treatment (7.5 mg per pellet 60 day release = 0.125 mg·day$^{-1}$, $n$ = 13) or placebo treatment ($n$ = 12). Mice were anaesthetized by use of isoflurane inhalation, 5% starting dose, 1.5% maintenance dose. The paws of the mice were pinched to verify the absence of a pain response. Pellets containing either haloperidol or empty placebo pellets (Innovative Research, Sarasota, FL, USA) were implanted s.c. between the shoulders, ensuring continuous drug release. One day after pellet implantation, mice were challenged with a Western-type diet containing 15% (w w$^{-1}$) total fat and 0.25% (w w$^{-1}$) cholesterol (Diet W; Special Diet Services, Witham, UK) to induce the development of atherosclerosis lesions. After 5 weeks of the Western-type diet feeding, mice were anaesthetized, bled by orbital bleeding and killed. Following perfusion of the arterial tree *in situ* with PBS, the heart and other organs were excised and fixed in 3.7% neutral-buffered formalin (Formalfixx; Shandon Scientific Ltd., Leicestershire, UK) or stored at –20°C until further analysis.

### Oral glucose tolerance test

Twenty-two days after the initiation of the Western-type diet, six mice of both treatment groups were subjected to an oral glucose tolerance test (OGTT). All mice were fasted after food withdrawal from 17:30 h the day before the test. The OGTT was started at 10:00 h. An initial blood sample ($t$ = 0) was taken, immediately followed by administration of 2 g·kg$^{-1}$ glucose in water, through an orogastric tube. Additional blood samples were taken via tail bleeding at 20, 40, 60, 90 and 120 min after glucose injection for measurement of plasma glucose levels with an Accu-Check glucometer (Roche Diagnostics, Almere, The Netherlands).

### Plasma lipid analysis

Cholesterol and triglycerides in plasma were determined using a standard enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany). The distribution of cholesterol over the different lipoproteins in plasma was determined by fractionation of a pool of 12 μL serum of three to four individual mice using a Superose 6 column (3.2 × 300 mm, Smart-system; Pharmacia, Uppsala, Sweden). Cholesterol content of the effluent was determined as indicated.

### Histological analysis of atherosclerosis

The arterial tree was perfused *in situ* with PBS (at a pressure of 100 mmHg) for 10 min via a cannula in the left ventricular apex. The heart plus aortic root was excised and stored in 3.7% neutral-buffered formalin (Formalfixx®; Shandon Scientific Ltd.). The atherosclerotic lesion areas in Oil red O-stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK). Mean lesion area (μm$^2$) was calculated from Oil red O-stained sections starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of macrophages using a rat MOMA-2 antibody, dilution 1:750 (Serotec Ltd, Oxford, UK). Goat anti-rat coupled to HRP (1:100) (Dako, Glostrup, Denmark) was used as a secondary antibody and Nova red substrate (Vector Laboratories, Burlingame, CA, USA) was used for HRP visualization. Collagen content of the lesions was visualized using Masson's Trichrome staining (Sigma Diagnostics, St Louis, MO, USA). TUNEL staining using the In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) was performed to determine the rate of apoptosis in lesional macrophages. TUNEL-positive nuclei were visualized with Nova Red (Vector Laboratories) and sections were counterstained with 0.3% methyl green. All quantifications were performed blinded by computer-aided morphometric analysis using the Leica image analysis system.

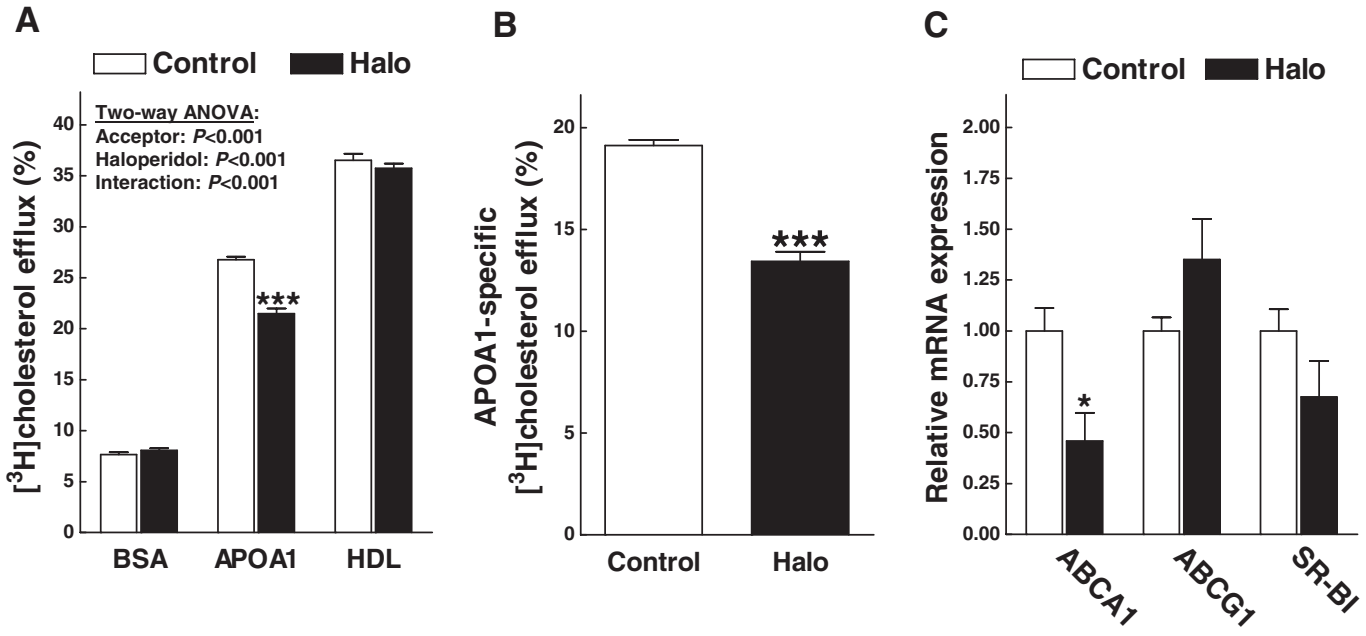
### Statistical analysis

Data are presented as means ± SEM. Statistical analysis was conducted using GraphPad Prism Software (Graphpad Software, La Jolla, CA, USA). Statistically significant differences among the means of the two treatment groups were tested using Student's two-tailed, unpaired $t$-test, or a two-way ANOVA with a Bonferroni *post* test to compare replicate means by row where appropriate. A probability value $P < 0.05$ was considered significant.

## Results

### Haloperidol treatment diminishes the ABCA1-mediated cholesterol efflux from macrophages in vitro

A potential effect of haloperidol on the macrophage cholesterol efflux function was evaluated in cultured murine thioglycollate-elicited peritoneal macrophages (Figure 1A). HDL was utilized as acceptor for cholesterol acquired by scavenger receptor BI (SR-BI) and ATP-binding cassette transporter G1 (ABCG1)-mediated transport, while the rate of [$^3$H]-cholesterol efflux to apolipoprotein A1 (APOA1) was considered a quantitative measure for the ABCA1 functionality. As expected, the acceptor availability had a significant impact on




## Figure 1

Effect of haloperidol treatment (Halo) on the cholesterol efflux function of peritoneal macrophages. (A) Cholesterol efflux to medium was measured in the presence (APOA1/HDL) or absence (BSA) of added acceptors. (B) Cholesterol efflux to APOA1 normalized for background efflux to BSA. (C) Relative mRNA expression levels of genes involved in cholesterol efflux. Data represent means + SEM. *$P < 0.05$, ***$P < 0.001$.

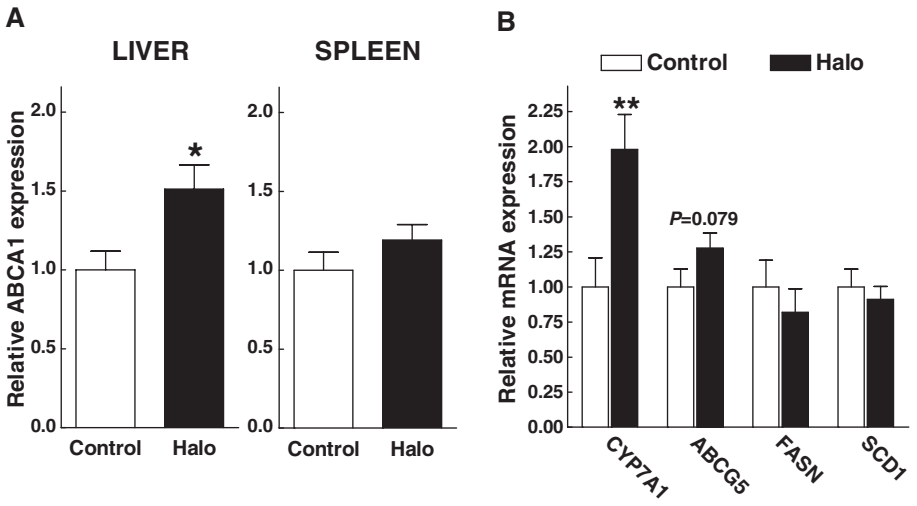
the cholesterol efflux rate (two-way ANOVA; $P < 0.001$). The accumulation of [$^3$H]-cholesterol in the culture medium of solvent control-treated cells was respectively 3.5- and 4.8-fold higher in the presence of 10 μg·mL$^{-1}$ APOA1 and 50 μg·mL$^{-1}$ HDL ($P < 0.001$ for both). Treatment with 10 μM haloperidol also significantly influenced the overall cholesterol efflux profile (two-way ANOVA; $P < 0.001$). Haloperidol treatment did not change the basal, acceptor-independent flux of cholesterol from cells or the HDL-induced cholesterol mobilization rate. In accordance, no significant change was noted in the relative mRNA expression levels of SR-BI and ABCG1 in response to haloperidol treatment (Figure 1C). Strikingly, the percentage of [$^3$H]-cholesterol in medium containing APOA1 was significantly lower (–20%; $P < 0.001$; Figure 1A) in haloperidol-treated cells as compared with dimethylsulfoxide control-treated cells. When considering the efflux percentage that can be solely attributed to the addition of APOA1 into the medium, it appears that the relative amount of cholesterol transported by ABCA1 was decreased by 30% ($P < 0.001$) upon haloperidol treatment (Figure 1B). As evident from Figure 1C, haloperidol treatment was associated with a parallel 54% decrease ($P = 0.043$) in the relative mRNA expression level of ABCA1. It can therefore be suggested that, in murine peritoneal macrophages, haloperidol diminishes the ABCA1 transcription rate and/or decreases ABCA1 mRNA stability and, as a result, lowers the efflux of cholesterol to APOA1.

### *Haloperidol treatment lowers the susceptibility for atherosclerosis* in vivo

To investigate whether the inhibitory effect of haloperidol on the macrophage cholesterol efflux function *in vitro* also translated into an altered atherosclerosis susceptibility *in vivo*, hyperlipidaemic LDL receptor knockout mice were implanted with an empty pellet (control) or a slow-release pellet containing haloperidol, s.c., and fed a Western-type diet enriched in cholesterol and fat for 5 weeks to induce the development of atherosclerotic lesions.

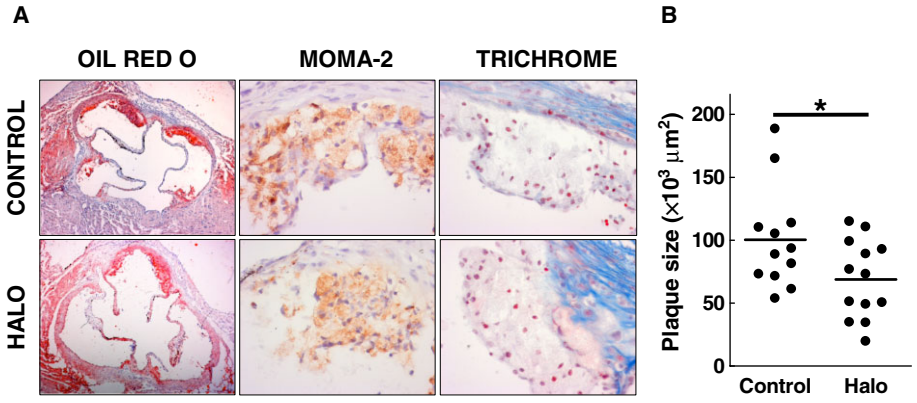
The effect of haloperidol on the mRNA expression of ABCA1 was determined in livers and spleens of the different mice as measures for the expression level in respectively hepatocytes and tissue macrophages (Figure 2A). A 51% increase ($P = 0.021$) in ABCA1 gene expression was noted in liver upon haloperidol treatment. In contrast, splenic relative mRNA expression levels of ABCA1 were not significantly changed by haloperidol exposure. The effect of haloperidol treatment on the *in vivo* expression of ABCA1 thus appears to be cell-type dependent.

The cell culture studies by Vik-Mo *et al.* (2009) have suggested that the nuclear receptor liver X receptor (LXR) can be involved in the effect of psychotropic drugs on metabolic gene expression *in vitro*. To evaluate the potential contribution of LXR in the effect of haloperidol on hepatocyte ABCA1 expression in our *in vivo* setting, we quantified the relative mRNA expression level of other established LXR target genes in livers of haloperidol-treated mice (Figure 2B). Expression of the sensitive LXR target gene cholesterol 7α-hydroxylase (CYP7A1) increased upon haloperidol treatment (+98%; $P = 0.004$). In addition, transcript levels of the hepatic canalicular half transporter ABCG5 tended to increase (+28%; $P = 0.079$). In contrast, no change was observed in the expression of the lipogenic LXR targets fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1). *In vivo* haloperidol treatment thus




## Figure 2

Effect of haloperidol treatment on relative expression levels of ABCA1 gene and other established LXR target genes in organs from Western-type diet-fed LDL receptor knockout mice. (A) Expression of ABCA1 in spleens and livers. (B) Hepatic mRNA expression levels of LXR target genes involved in cholesterol transport and lipogenesis. Data represent means + SEM. *$P < 0.05$, **$P < 0.01$.




## Figure 3

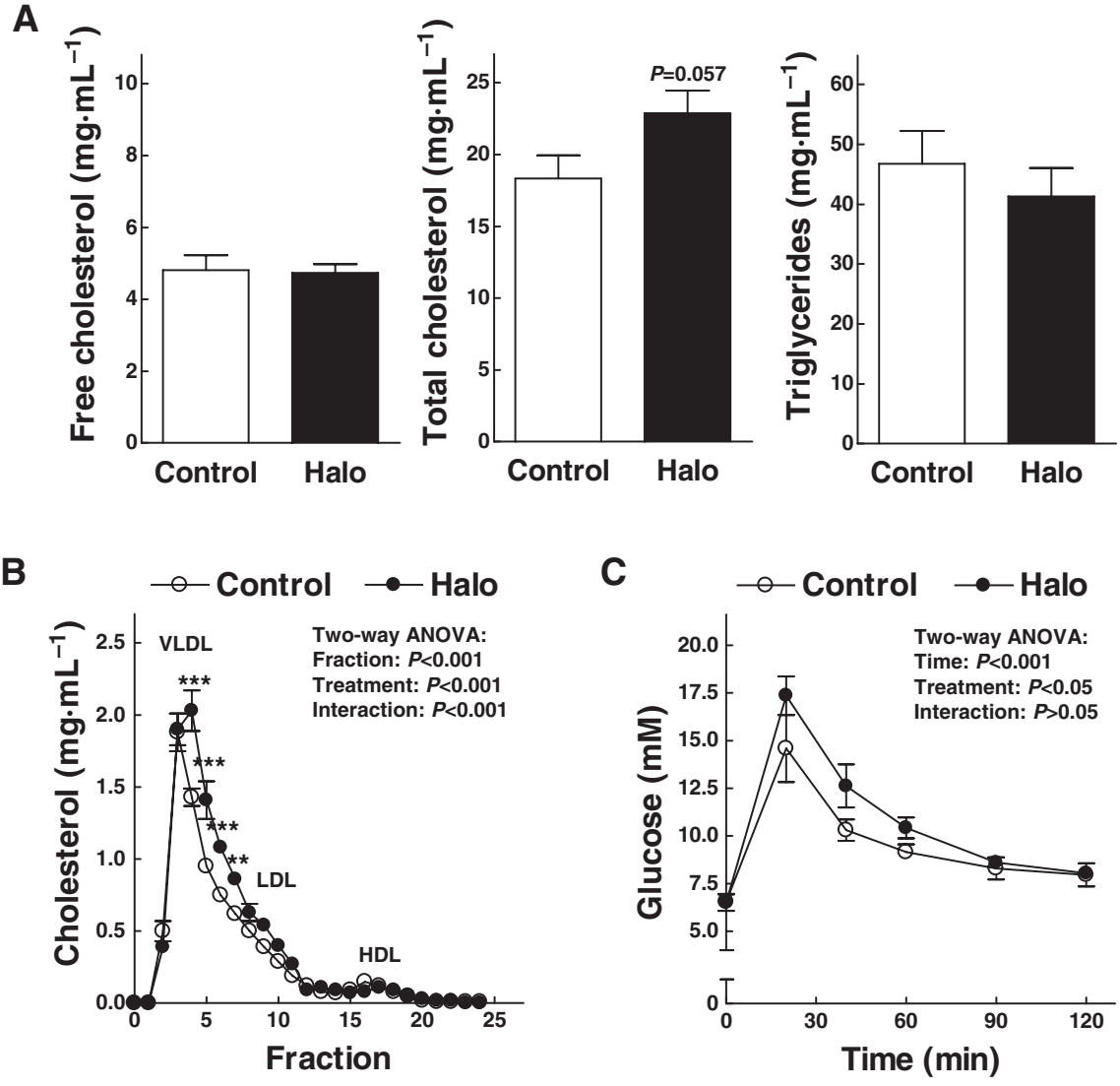
Representative pictures and the quantification of the size of aortic root atherosclerotic lesions in Western-type diet-fed LDL receptor knockout mice implanted with an empty (control) or haloperidol-containing (Halo) pellet. (A) Atherosclerotic lesions are visualized by Oil red O neutral lipid staining. Lesions were stained with MOMA-2 for macrophages (red/brown) and trichrome for collagen (blue) content. Macrophages can also be identified in trichrome-stained sections from their light purple/grey appearance and pink nuclei. (B) Quantification plot in which separate data points represent the lesion sizes of individual mice. Horizontal lines depict the respective group averages. *$P < 0.05$.

selectively stimulates the expression of LXR target genes involved in reverse cholesterol transport, but not lipogenesis, in hepatocytes.

Hearts and aortas were isolated after the mice had been killed and subsequently evaluated for the extent of atherosclerosis. Microscopic examination of the aortic specimens indicated that all mice lacked lesions in the aortic arch and the descending part of their aorta. This can probably be attributed to the relatively short period of Western-type diet feeding (5 weeks). In accordance with an in-general initial stage of lesion development, atherosclerotic plaques within the aortic root of control (empty) pellet-implanted mice consisted purely of macrophages (100% of cells MOMA-2 positive) and virtually lacked collagen (Figure 3A). Aortic root atherosclerotic lesions in haloperidol-treated mice exhibited a similar early stage appearance (Figure 3A). Furthermore, the relative number of apoptotic cells in the atherosclerotic plaques was not different between the two groups of mice as judged from TUNEL stainings (16 ± 3% apoptotic nuclei in haloperidol-treated vs. 14 ± 4% in empty pellet-implanted mice; $P > 0.05$). However, quantification of the aortic root plaque load revealed that the average atherosclerotic lesion size was significantly smaller (–31%; $P = 0.039$) in the haloperidol treatment group as compared with the control group (Figure 3B). Combined, these data suggest that haloperidol decreases the *in vivo* atherosclerosis susceptibility in an ABCA1-independent manner.

### *Haloperidol treatment is associated with a mildly more atherogenic metabolic profile*

We tried to uncover the mechanism behind the lower plaque load after haloperidol treatment. Haloperidol treatment has previously been associated with both disturbances in lipid and glucose homeostasis in the clinical setting (Perez-Iglesias *et al.*, 2007; 2009). Because metabolic changes can underlie an altered susceptibility to atherosclerosis, we measured plasma lipid levels and total body glucose tolerance. Plasma triglyceride and free cholesterol levels were not different between the two experimental groups (Figure 4A). However, total cholesterol levels tended to be higher (+25%; $P = 0.057$) in plasma of haloperidol-treated mice (Figure 4A). Fast performance liquid chromatography-based lipoprotein distribution analysis revealed that the change in total cholesterol levels could be attributed to a significant increase (two-way ANOVA: $P < 0.001$ for haloperidol effect) in the amount of cholesterol associated with pro-atherogenic VLDL/LDL fractions (Figure 4B). In contrast, no change was observed in plasma HDL-cholesterol levels. Fasting glucose levels were not altered by haloperidol exposure. In contrast, a significant increase in the overall plasma glucose response to an oral glucose load was noted in haloperidol-treated mice (two-way ANOVA: $P < 0.05$ for haloperidol effect; Figure 4C). Given the small increase in pro-atherogenic VLDL/LDL-cholesterol levels and the parallel decrease in glucose tolerance, it is anticipated that the change in metabolic status induced by haloperidol treatment does not underlie the associated decrease in atherosclerosis susceptibility.




## Figure 4

Effect of haloperidol treatment on metabolic parameters measured in the plasma of Western-type diet-fed LDL receptor knockout mice. (A) Non-fasting lipid levels. (B) Cholesterol distribution over the different lipoproteins. Fractions 1–12 represent VLDL/LDL and fractions 13–20 represent HDL. (C) Time course of glucose levels in response to an oral glucose dose. All data represent means + SEM.

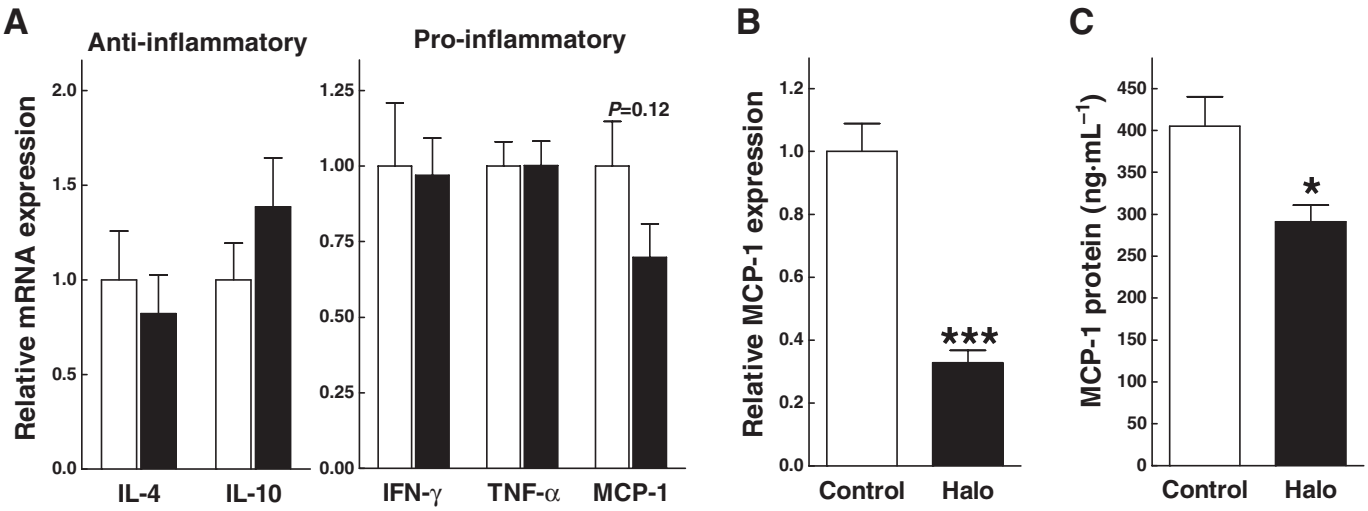
### *Haloperidol treatment lowers macrophage monocyte chemotactic protein-1 (MCP-1) expression*

Inflammation, that is, sub-endothelial infiltration and entrapment of monocytes and other immune cells, is an essential part of the atherosclerosis pathology. Recent studies have indicated that the hyperprolactinaemia associated with haloperidol treatment inhibits inflammation in a rat model of rheumatoid arthritis (Adán *et al.*, 2013). We therefore further examined the potential effect of *in vivo* haloperidol treatment on the overall immune status. Total white blood cell numbers and relative spleen weights were not altered by haloperidol treatment. In addition, haematological analysis did not indicate a difference in the absolute concentration of all major leukocyte sub-populations in the blood of the two groups of mice (data not shown). Splenic gene expression levels of anti-inflammatory IL-4 and IL-10 were also not significantly altered (Figure 5A). Furthermore, mRNA expression levels of the pro-inflammatory cytokines TNF-α and IFN-γ levels were virtually identical in spleens of both experimental groups (Figure 5A). A clear trend towards a decrease (–30%; $P = 0.12$) in the relative expression level of monocyte chemotactic protein-1 (MCP-1/CCL2), however, was detected in spleens of haloperidol-treated mice (Figure 5A). The heterogeneity of the spleen regarding the cell content, that is, monocytes/macrophages and T- and B-cells, can possibly explain the failure of the MCP-1 effect to become significant. *In vitro* studies in cultured peritoneal macrophages were therefore performed to show a potential direct effect of haloperidol on the expression levels and secretion rate of MCP-1. As can be appreciated from Figure 5B, exposure to 10 μM of haloperidol for 24 h resulted in a striking 70% reduction ($P < 0.001$) in MCP-1 transcript levels. More importantly, this was paralleled by a 28% decrease ($P = 0.018$) in the secretion of MCP-1 protein from the cells into the culture medium (Figure 5C). Based upon these observations, it is thought that haloperidol inhibits the chemotactic potential of macrophages both *in vitro* and *in vivo*.

## Discussion and conclusions

The major finding of our current studies is that haloperidol treatment is able to protect against atherosclerosis in spite of a mildly more atherogenic metabolic profile. In accordance with a potent atheroprotective capacity of haloperidol that is independent of beneficial effects on the plasma compartment, earlier studies by Houser *et al.* (2000) have also shown a decrease in atherosclerotic plaque burden without parallel changes in serum cholesterol or triglyceride levels upon haloperidol treatment in rats.

Our studies clearly show that the beneficial effect of haloperidol on atherosclerotic lesion development can not be attributed to an increase in macrophage ABCA1 expression or and/cholesterol efflux potential. In accordance with the data from Vik-Mo *et al.* (2009) which showed the potency of haloperidol to up-regulate ABCA1 in the human HepG2 hepatoma cell line, haloperidol did increase hepatic ABCA1 expression in our hyperlipidaemic LDL receptor knockout mouse model. However, no significant effect on splenic (macrophage) ABCA1 expression was noted *in vivo*, while haloperidol treatment decreased ABCA1 expression and the cholesterol efflux to APOA1 in cultured macrophages *in vitro*. Interestingly, a critical review of the data by Vik-Mo *et al.* (2009) showed that, among the psychotic drugs evaluated, haloperidol specifically decreased ABCA1 expression also in human glioblastoma cells. Given the overlapping functions of macrophages and certain types of glia cells, that is, microglia, we anticipate that effects of haloperidol treatment on the regulation of gene transcription noted in *in vitro* studies can, in general, be efficiently translated into the *in vivo* setting.




## Figure 5

Effect of *in vitro* and *in vivo* treatment with haloperidol on the expression and/or secretion of pro- and anti-inflammatory cytokines. (A) Splenic relative mRNA expression levels of the indicated cytokines. (B) Peritoneal macrophage MCP-1 expression levels. (C) MCP-1 protein levels measured in cell culture medium of peritoneal macrophages. Data represent means + SEM. *$P < 0.05$, ***$P < 0.001$.

We have tried to delineate the mechanism through which haloperidol inhibits atherogenesis. MCP-1 expression levels were markedly decreased in *in vitro* cultured macrophages and also tended to decrease in the spleen, an established macrophage-rich tissue, upon haloperidol exposure *in vivo*. MCP-1 interacts with the chemokine receptor CCR2 on monocytes to facilitate monocyte recruitment. Disruption of the MCP-1/CCR2 axis has been shown to slow down the initiation of atherosclerosis in several types of hyperlipidaemic mice (reviewed by Charo and Taubman, 2004), which highlights the importance of MCP-1 function for the development of atherosclerotic lesions. We therefore consider the haloperidol-induced decrease in macrophage chemotaxis the primary reason for the observed protection against atherosclerosis.

The increase in hepatic ABCA1 expression in haloperidol-treated mice was paralleled by similar changes in other known LXR target genes involved in reverse cholesterol transport and biliary cholesterol disposal such as CYP7A1 and ABCG5. As also previously suggested by Vik-Mo *et al.* (2009), haloperidol may therefore in hepatocytes directly impact on LXR-mediated gene regulation, resulting in a general stimulation of LXR target genes. Activation of LXR by the synthetic ligands T0901317 and GW3965 not only efficiently lowers atherosclerotic plaque burden (Joseph *et al.*, 2002; Terasaka *et al.*, 2003; Chen *et al.*, 2012) but also induces major metabolic side effects in liver, that is, hypertriglyceridaemia (Schultz *et al.*, 2000) and fatty liver development (Grefhorst *et al.*, 2002). Interestingly, we did not observe a change in the established lipogenic LXR-sensitive genes FASN and SCD1 in livers of haloperidol-treated mice. Haloperidol treatment apparently induces gene-selective modulation of LXR targets in hepatocytes *in vivo*. The fact that we did not note a change in plasma triglyceride levels upon haloperidol treatment further supports the absence of functional changes in LXR-mediated lipogenic pathways. It can therefore be anticipated that haloperidol – in addition to the recently identified LXR ligands 2,4,6-trihydroxybenzoate (Hoang *et al.*, 2012b) and fucosterol (Hoang *et al.*, 2012a) – should be added to the list of selective LXR modulators that only stimulate the hepatic expression of genes involved in reverse cholesterol transport.

A note of caution is necessary before translating our current findings to the human (clinical) setting and providing cardiovascular disease patients with haloperidol. Although haloperidol is generally well tolerated in humans, it should be acknowledged that multiple studies have identified corrected QT (QTc) interval prolongation as side effect of haloperidol use, especially in combination treatments (Metzger and Friedman, 1993; Tisdale *et al.*, 2001). An increase in QTc interval is causally related to total mortality and cardiac sudden death (van der Bijl *et al.*, 2012; Noseworthy *et al.*, 2012). Furthermore, high-risk patients are generally prescribed with other drugs, that is, lipid-lowering pharmaceuticals such as statins and/or blood pressure lowering agents. We therefore do not recommend haloperidol use for the treatment of cardiovascular disease, but suggest that haloperidol can rather be used as model compound in studies aimed at defining the molecular mechanism underlying LXR's gene selectivity in hepatocytes.

In conclusion, our studies show that haloperidol treatment lowers the susceptibility of hyperlipidaemic LDL receptor knockout mice to develop atherosclerotic lesions. Our findings suggest that the beneficial effect of haloperidol on atherosclerosis susceptibility can be attributed to its ability to inhibit macrophage chemotaxis.

## Acknowledgements

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## Author contributions

M. H., A. Q. R., J. E. N. and R. J. v. d. S. designed and performed the research. M. H. and R. J. v. d. S. analysed the data and wrote the paper. M. V. E., as head of the lipid group within the Division of Biopharmaceutics of LACDR, facilitated the research.

## Conflict of interest

The authors have nothing to disclose.

## References

Adán N, Guzmán-Morales J, Ledesma-Colunga MG, Perales-Canales SI, Quintanar-Stéphano A, López-Barrera F *et al.* (2013). Prolactin promotes cartilage survival and attenuates inflammation in inflammatory arthritis. J Clin Invest 123: 3902–3913.

Aiello RJ, Brees D, Bourassa PA, Royer L, Lindsey S, Coskran T *et al.* (2002). Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. Arterioscler Thromb Vasc Biol 22: 630–637.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: Nuclear hormone receptors. Br J Pharmacol 170: 1652–1675.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Transporters. Br J Pharmacol 170: 1706–1796.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013c). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

van der Bijl P, Heradien M, Doubell A, Brink P (2012). QTc prolongation prior to angiography predicts poor outcome and associates significantly with lower left ventricular ejection fractions and higher left ventricular end-diastolic pressures. Cardiovasc J Afr 23: 541–545.

Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB *et al.* (1977). HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation 55: 767–772.

Charo IF, Taubman MB (2004). Chemokines in the pathogenesis of vascular disease. Circ Res 95: 858–866.

Chen J, Zhao L, Sun D, Narsinh K, Li C, Zhang Z *et al.* (2012). Liver X receptor activation attenuates plaque formation and improves vasomotor function of the aortic artery in atherosclerotic ApoE(–/–) mice. Inflamm Res 61: 1299–1307.

Cholesterol Treatment Trialists' (CTT) Collaborators, Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J *et al.* (2012). The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. Lancet 380: 581–590.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159.

Grefhorst A, Elzinga BM, Voshol PJ, Plösch T, Kok T, Bloks VW *et al.* (2002). Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J Biol Chem 277: 34182–34190.

Hoang MH, Jia Y, Jun HJ, Lee JH, Lee BY, Lee SJ (2012a). Fucosterol is a selective liver X receptor modulator that regulates the expression of key genes in cholesterol homeostasis in macrophages, hepatocytes, and intestinal cells. J Agric Food Chem 60: 11567–11575.

Hoang MH, Jia Y, Jun HJ, Lee JH, Lee DH, Hwang BY *et al.* (2012b). Ethyl 2,4,6-trihydroxybenzoate is an agonistic ligand for liver X receptor that induces cholesterol efflux from macrophages without affecting lipid accumulation in HepG2 cells. Bioorg Med Chem Lett 22: 4094–4099.

Hoekstra M, Kruijt JK, Van Eck M, Van Berkel TJ (2003). Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. J Biol Chem 278: 25448–25453.

Houser SL, Aretz HT, Quist WC, Chang Y, Schreiber AD (2000). Serum lipids and arterial plaque load are altered independently with high-dose progesterone in hypercholesterolemic male rabbits. Cardiovasc Pathol 9: 317–322.

Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA *et al.* (2002). Synthetic LXR ligand inhibits the development of atherosclerosis in mice. Proc Natl Acad Sci U S A 99: 7604–7609.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Metzger E, Friedman R (1993). Prolongation of the corrected QT and Torsades de pointes cardiac arrhythmia associated with intravenous haloperidol in the medically ill. J Clin Psychopharmacol 13: 128–132.

Noseworthy PA, Peloso GM, Hwang SJ, Larson MG, Levy D, O'Donnell CJ *et al.* (2012). QT interval and long-term mortality risk in the Framingham Heart Study. Ann Noninvasive Electrocardiol 17: 340–348.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.*; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–D1106.

Perez-Iglesias R, Crespo-Facorro B, Amado JA, Garcia-Unzueta MT, Ramirez-Bonilla ML, Gonzalez-Blanch C *et al.* (2007). A 12-week randomized clinical trial to evaluate metabolic changes in drug-naive, first-episode psychosis patients treated with haloperidol, olanzapine, or risperidone. J Clin Psychiatry 68: 1733–1740.

Perez-Iglesias R, Mata I, Pelayo-Teran JM, Amado JA, Garcia-Unzueta MT, Berja A *et al.* (2009). Glucose and lipid disturbances after 1 year of antipsychotic treatment in a drug-naïve population. Schizophr Res 107: 115–121.

Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L *et al.* (2000). Role of LXRs in control of lipogenesis. Genes Dev 14: 2831–2838.

Terasaka N, Hiroshima A, Koieyama T, Ubukata N, Morikawa Y, Nakai D *et al.* (2003). T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. FEBS Lett 536: 6–11.

Tisdale JE, Rasty S, Padhi ID, Sharma ND, Rosman H (2001). The effect of intravenous haloperidol on QT interval dispersion in critically ill patients: comparison with QT interval prolongation for assessment of risk of Torsades de Pointes. J Clin Pharmacol 41: 1310–1318.

Van Eck M, Bos IS, Kaminski WE, Orsó E, Rothe G, Twisk J *et al.* (2002). Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. Proc Natl Acad Sci U S A 99: 6298–6303.

Van Eck M, Singaraja RR, Ye D, Hildebrand RB, James ER, Hayden MR *et al.* (2006). Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. Arterioscler Thromb Vasc Biol 26: 929–934.

Vik-Mo AO, Fernø J, Skrede S, Steen VM (2009). Psychotropic drugs up-regulate the expression of cholesterol transport proteins including ApoE in cultured human CNS- and liver cells. BMC Pharmacol 9: 10.