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# Cholesterol modulates LRP5 expression in the vessel wall

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ABSTRACT

*Objective:* Macrophages are key players in atherosclerotic lesion formation and progression. We have recently demonstrated that lipid-loaded macrophages show activation of the canonical Wnt signaling pathway.
*Methods:* To test the *in vivo* role of the canonical Wnt pathway in atherosclerosis we used mice deficient in the Wnt signaling receptor LRP5 ($LRP5^{-/-}$) fed a hypercholesterolemic diet (HC) to induce atherosclerosis. These dietary groups were further subdivided into two subgroups receiving their respective diets supplemented with 2% plant sterol esters (PSE). All mice remained on their assigned diets until age 18 weeks.
*Results:* HC WT mice had mildly increased non-HDL cholesterol levels, developed aortic atherosclerotic lesions and showed upregulated expression levels of aortic Lrp5. HC $LRP5^{-/-}$ mice develop larger aortic atherosclerotic lesions than WT mice indicating that LRP5 has a protective function in atherosclerosis progression. The oral administration of PSE, a dietary cholesterol-lowering agent, had an effect in the expression levels of the Wnt signaling receptor and in atherosclerosis progression. We found that PSE reduced serum total cholesterol levels, abolished HC-induced LRP5 overexpression and reduced aortic atherosclerotic plaques.
*Conclusion:* The proatherogenic effects of the excess of plasma lipids are in part mediated by modulation of LRP5 in the aorta. LRP5 and canonical Wnt signaling exert a protective defense mechanism against hyperlipidemia and atherosclerosis lesion progression.


## 1. Introduction

Atherosclerotic lesions are formed by the accumulation of lipids and fibrous elements in large and medium size arteries in response to injury [1]. Under physiological conditions, cells store fatty acids and fatty alcohols in the form of neutral lipids (triacylglycerols, TAG and cholesteryl esters, CE) that are used for membrane formation and energy supply [2]. However, elevated cytoplasmic deposition of neutral lipids is a significant risk factor for several pathologies. For example, the accumulation of CE in smooth muscle cells and macrophages in the vessel wall comprises the earliest recognizable stage in atherosclerotic plaque formation [2]. Furthermore, serum levels of TAG and total cholesterol are independent risk factors for atherosclerosis. High low-density lipoprotein plasma levels (LDL) are also considered risk factors for atherosclerosis [3–5]. Atherosclerotic plaques are characterized by the accumulation of lipid-loaded foam cells, increased levels of oxidized-LDL, HDL, TAG, phospholipids, and oxysterols, and the accumulation of fibrinogen, apo-AI, clusterin and paraoxonase [6–8]. Lipid droplets in the intima wall contain about half the lipids in the lesions with a composition of 95% CE, 1.5% free cholesterol, 1% phospholipids and 2,5% TAG [9].

Lipid internalization in the vascular wall is mediated by the LDL superfamily of receptors including LDLR, VLDLR, LDL receptor-related proteins (LRPs) and scavenger receptors [10]. Our group showed that two LRPs, LRP1 and LRP5 are involved in lipid uptake leading to an increase in intracellular CE accumulation in human vascular smooth muscle cells and macrophages [11,12].

LRP5 is a receptor of the canonical Wnt/β-catenin pathway. LRP5 involvement in the modulation of glucose-induced insulin secretion was shown with $LRP5^{-/-}$ mice that had a decreased glucose-induced insulin secretion as a result of reduced cellular ATP and calcium levels in pancreatic islets [13]. LRP5 involvement in cholesterol metabolism comes from the observation that $ApoE^{-/-}LRP5^{-/-}$ mice have larger atherosclerotic lesions

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than their ApoE$^{-/-}$ littermates [14]. However, the exceedingly high levels of plasma cholesterol in ApoE$^{-/-}$LRP5$^{-/-}$ mice (almost 750 mg/dl) could have shadowed any effect of LRP5.

Several studies have evaluated the use of plant sterol esters (PSE) as a cholesterol-lowering strategy [15–18]. PSE reduce cholesterol absorption by competitively inhibiting its internalization and processing in enterocytes in the intestine [18,19]. Although the beneficial cardiovascular effects of PSE are mainly because their plasma lipid-lowering capacity, they can decrease growth and proliferation of smooth muscle cells and reduce foam cell formation [20]. PSE can also attenuate the inflammatory response by ameliorating endothelial dysfunction in ApoE$^{-/-}$ mice [21] and decrease aggregated LDL-induced secretion of pro-inflammatory cytokines (TNFα, IL-6 and IL1B) in cultured macrophages [22]. Furthermore, plant sterol feeding have been shown to have anti-inflammatory properties in different cohorts of healthy subjects although its association with reduced cholesterol absorption was not determined [17,23,24].

Our previous results show that LRP5 is overexpressed in cultured lipid-loaded macrophages [12]. Here, we investigated whether the modulation of plasma lipid levels by cholesterol-lowering strategies can modulate the canonical Wnt signaling receptor and cholesteryl ester content in aortic atherosclerotic plaques *in vivo*.

## 2. Materials and methods

### 2.1. Experimental design

LRP5$^{-/-}$ mice, a kind gift from Dr. Bart Williams [25–27] were maintained in a C57BL/6 background. Mice were housed in cages under controlled temperature (21 ± 2 °C) on a 12 h light/dark cycle with food and water *ad libitum*. Homozygous wild-type C57BL/6 mice (WT; $n = 34$) and LRP5$^{-/-}$C57BL/6 mice (LRP5$^{-/-}$; $n = 34$) were used for the protocols. The presence of LRP5 alleles was assessed by PCR amplification from DNA extracted from tail biopsies in wild type, heterozygous and homozygous littermates. Primers used were S17 (GGC TCG GAG GAC AGA CCT GAG), S23 (CTG TCA GTG CCT GTA TCT GTC C) and IRES31 (AGG GGC GGA ATT CGA TAG CT). LRP5$^{-/-}$ and WT mice were fed a normal chow diet (NC, Tekland diet, Harland Labs) for 10 weeks. Animals were then divided into 3 groups to be fed with NC diet, high cholesterol diet (HC, TD.88137, Harland Labs) or HC diet supplemented with 2% Plant Sterol Esters (w/w) for further 8 weeks ($n = 8–12$/group). PSE were provided by Danone (Barcelona, Spain). All sterol analyses were performed by gas liquid chromatography. The study protocol was approved by the local institutional animal research committee (ICCC051/5422) and was performed in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### 2.2. Biochemical analysis

Blood samples were collected in serum separator gel tubes. Serum was obtained by centrifugation 3500 rpm, 20 min at 4 °C. Cholesterol, triglycerides and HDL levels were measured enzymatically by using commercially available kits (GERNON reagents) and read in a spectrophotometer (MC-15 SOFT; RAL).

### 2.3. Quantification of atherosclerotic lesions

Mice were anesthetized and aortas were removed, carefully cleaned of adventitial fat under a stereoscopic microscope and longitudinally cut with the luminal surface facing up ($n = 6–12$ mice/group). Aortas were fixed overnight in 4% paraformaldehyde, washed with dd$H_2O$ 1 h in gently shaking and stained with Oil-red-O (ORO) for 30 min. Aortas were rinsed with 70% ethanol and dd$H_2O$; images were captured by Nikon Eclipse 80i microscope and digitized by Retiga 1300i Fast camera. ORO-stained area was quantified with Image J software and results are expressed as percentage of lipid area/total aortic area.

### 2.4. Thin layer chromatography (TLC)

Aortas (5 mg) were homogenized in NaOH 0.1 M. The organic solvent was removed under a $N_2$ stream; the lipid extract was suspended in dichloromethane and TLC was performed on silica G-24 plates. Different concentrations of standards (a mix of cholesterol, cholesterol palmitate, triglycerides, diglycerides and monoglycerides) were applied to each plate. The chromatographic developing solution was heptane/diethyl ether/acetic acid (74:21:4, vol/vol/vol). The spots corresponding to cholesteryl esters (CE), triacylglycerides (TAG), diacylglycerides (DAG), monoacylglycerides (MAG) and free cholesterol (FC) were quantified by densitometry.

### 2.5. Real-time RT-PCR

Aortas were frozen in liquid nitrogen and aortic RNA was isolated with Trizol® Reagent (Invitrogen) ($N = 5–8$ mice/group). Concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and purity was checked by the A260/A280 ratio (ratios between 1.8 and 2.1 were considered acceptable). cDNA was synthesized from 0.5 μg RNA with cDNA Reverse transcription kit (Qiagen). The resulting cDNA samples were amplified with a with RT-PCR thermal cycler (Applied Biosystems 7900HT) and LRP5 (Mm.PT.49a.8045420) and LDLR (Mm.PT.49a.9930556) specific probes. Results were normalized with 18S probe from Applied Biosystems.

### 2.6. Statistical analysis

Results are expressed as mean ± S.E.M. A Stat View statistical package was used for all the analysis. When possible, comparisons among groups were performed by parametric analysis (one factor ANOVA). Statistical significance was considered when $p < 0.05$.

## 3. Experimental results

### 3.1. Hypercholesterolemic diet increases serum cholesterol and aortic lipid deposition

To determine whether cholesterol-lowering strategies modulated serum cholesterol profile in LRP5$^{-/-}$ mice, WT and LRP5$^{-/-}$ mice were fed a hypercholesterolemic (HC) diet supplemented or not with 2% plant sterol esters (HC + PSE). A normocholesterolemic (NC) control group was also analyzed. Diet composition details are shown in Table 1.

Serum levels of total cholesterol, HDL and non-HDL were significantly increased in HC WT mice and HC LRP5$^{-/-}$ mice with respect to their NC littermates (Sup. Table 1). Plant sterol esters supplementation to HC diets in WT mice reduced total cholesterol and non-HDL-C serum concentrations without affecting HDL-C levels as previously described [28]. However, PSE feeding in LRP5$^{-/-}$ mice induced an increase in HDL-C serum levels respect to HC LRP5$^{-/-}$ mice. Serum triglycerides (TAG) concentrations were reduced after PSE + HC diets with respect to HC diets in WT mice while TAG levels did not differ significantly among dietary treatment groups in LRP5$^{-/-}$ mice (Sup. Table 1).