Increased HDL cholesterol and apo A-I in humans and mice treated with a novel SR-BI inhibitor

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Abstract

Objectives—Increasing HDL levels is a potential strategy for the treatment of atherosclerosis.

Methods and results—ITX5061, a molecule initially characterized as a p38 MAPK inhibitor, increased HDL-C levels by 20 % in a human population of hypertriglyceridemic subjects with low HDL levels. ITX5061 also moderately increased apoA-I but did not affect VLDL/LDL cholesterol or plasma triglyceride concentrations. ITX5061 increased HDL-C in WT and human apoA-I transgenic mice and kinetic experiments showed that ITX5061 decreased the fractional catabolic rate of HDL-CE and reduced its hepatic uptake. In transfected cells, ITX5061 inhibited SR-BI-dependent uptake of HDL-CE. Moreover, ITX5061 failed to increase HDL-C levels in $SR\text{-}BI^{-/-}$ mice. To assess effects on atherosclerosis, ITX5061 was given to atherogenic diet-fed $Ldlr^{+/-}$ mice with or without CETP expression for 18 weeks. In both the control and CETP-expressing groups, ITX5061 treated mice displayed reductions of early atherosclerotic lesions in the aortic arch (−40 %, p<0.05), and a non-significant trend to reduced lesion area in the proximal aorta.

Conclusions—Our data indicate that ITX5061 increases HDL-C levels by inhibition of SR-BI activity. This suggests that pharmacological inhibition of SR-BI has the potential to raise HDL-C and apoA-I levels without adverse effects on VLDL/LDL cholesterol levels in humans.

Keywords

Scavenger Receptor B-I; High Density Lipoproteins; Inhibitors; p38 MAPK; atherosclerosis

Introduction

Plasma high-density lipoprotein (HDL) levels are inversely correlated with atherosclerotic cardiovascular disease and raising HDL levels by lifestyle changes or pharmacological interventions is an emerging strategy that might help to reduce the residual burden of disease in patients treated with Low-density lipoprotein (LDL) lowering approaches [1, 2]. While increasing synthesis or infusion of apolipoprotein A-I (apoA-I) or HDL reduces atherosclerosis

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in animals and humans, it is not clear that all approaches to raising HDL will reduce atherosclerosis.

The scavenger receptor B-I (SR-BI) is a major factor regulating HDL catabolism. SR-BI binds HDL and mediates the selective uptake of HDL-cholesteryl ester (CE) in the liver and steroidogenic tissues. [3]. While the role of SR-BI in HDL metabolism in mice and rats has been clearly shown, much less information is available on the function of SR-BI in humans. Primary human hepatocytes do demonstrate selective uptake of HDL-CE [4] but the quantitative importance of this pathway has not been shown. In humans, cholesteryl ester transfer protein (CETP), a factor lacking in mice and rats, plays a major role in HDL-CE catabolism. In the present study, we demonstrate that ITX5061, a molecule initially characterized as a p38 mitogen-actiated protein kinase (MAPK) inhibitor, caused an increase in HDL cholesterol (HDL-C) and apoA-I levels in humans. Studies in mice and cultured cells indicated that ITX5061 is an inhibitor of the SR-BI activity. Interestingly, ITX5061 did not cause increases in very-low-density lipoprotein (VLDL) and LDL cholesterol and reduced atherosclerosis in the aortic arch in LDL receptor (Ldlr) heterozygous mice fed an atherogenic diet.

Materials and methods

A complete Materials and Methods section is available in the online supplemental materials (online at http://atvb.ahajournals.org).

Molecule

Structure of ITX5061 is shown in figure S1. The empirical formula of ITX5061 is $C_{30}H_{38}ClN_3O_7S$. The molecular weight of ITX5061 (as an HCl salt) is 620.2.

Human data

KC706-C06 study was conducted from 6 November 2006 to 8 May 2007. It was a multicenter, randomized, double-blind, placebo-controlled study. Its primary objective was to evaluate the ability of ITX5061 to increase HDL-C in patients with low HDL-C and elevated triglycerides (TG) at Baseline.

Animals—C57Bl/6 Wild Type (WT) mice, C57Bl/6 SR-BI deficient mice (*SR-BI*$^{-/-}$) [5], and C57Bl/6 transgenic mice expressing human apoA-I under the control of its natural flanking regions (HuAITg) [6] were used in the present study. Atherosclerosis studies were conducted in F1 hybrid C57BL/6 × DBA/1 *Ldlr*$^{+/-}$ mice fed the Paigen diet, which is high-fat/cholesterol/ bile salt diet containing 1.25% cholesterol, 7.5% cocoa butter, and 0.5% cholic acid (TD 88051; Harlan Teklad, Madison, WI). Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Results

ITX5061 increases HDL levels in humans

ITX5061 was initially characterized as a type II (non competitive) inhibitor of p38 MAPK (Fig S2), and was noted to cause increases in HDL-C levels in humans. In order to systematically investigate effects on plasma lipoprotein levels, ITX5061 was administered at two different doses in a population of subjects with low HDL-C (men <45 mg/dl and women <55 mg/dl) and increased TG concentrations (TG ranging from 150 to 400 mg/dl). Baseline characteristics are given in table SI. Subjects received either placebo, ITX5061 at 150 mg or 300 mg daily. ITX5061 treatment resulted in an increase in HDL-C concentration that was similar in the two ITX5061 treated groups (approx. 20 % increases). The rise in HDL-C was observed throughout the treatment period and was reversed after cessation of the drug (Fig 1). The mean HDL

particle size was significantly increased in ITX5061 treated subjects (P<0.05) (Fig 2A). No significant changes in other lipid parameters (LDL-C and TG) were observed, although a transient, non-significant, increase in TG was observed after 2 weeks of treatment (Fig S3). ApoA-I protein levels were moderately increased in the 150 mg group (+ 10%, P<0.05) but were not statistically different in the 300 mg group (Fig 2B). Adverse events were similar in all groups except for one patient in the 150 mg group and 4 patients in the 300 mg group who had reversible increases in transaminase levels. These findings indicate a selective increase in HDL-C and apoA-I in subjects treated with the 150 mg dose of ITX5061. Further increases in these parameters were not seen at the 300 mg dose.

ITX5061 increases HDL-C and size in HuAITg mice

To investigate the mechanism of HDL-C raising by ITX5061, further studies were conducted in mice. HuAITg mice were treated with ITX5061 (30 mg/kg/day) or vehicle for one week. This resulted in a 50% increase in HDL-C levels compared to baseline, but no change in non-HDL-C levels (Fig 3A). As determined by scanning SDS-PAGE gels, apoA-I levels were moderately (+15 %) but significantly increased in ITX5061-treated HuAITg mice, compared to mice received vehicle (Fig 3B). Consistent with the more prominent increase in HDL-C than apoA-I, ITX5061 treatment induced a shift towards larger sized HDL as shown by native-PAGE (Fig 3C). In order to assess whether ITX5061 could affect expression of genes involved in HDL biosynthesis or catabolism in the liver, relative mRNA levels of apoA-I, ABCA1 and SR-BI were determined in ITX5061 and vehicle treated animals. No changes in mRNA levels were found for any of these transcripts (Fig S4) suggesting that ITX5061 raised HDL independent of these transcript levels.

ITX5061 decreases HDL-CE catabolism and hepatic uptake

To gain more insight into the mechanisms by which ITX5061 increases HDL-C, a kinetic study was performed. HDL labelled with [^{3}H] CE was injected via the tail vein into HuAITg mice pre-treated or not with ITX5061. As shown in Fig 3D, ITX5061 significantly decreased HDL-CE catabolism with an FCR of 1.86±0.40 pools/d vs 2.47±0.26 pools/d in the control group (P<0.05), while calculated production rates were identical in both groups (129±24 μg/g/d vs 129±16 μg/g/d). Moreover, accumulation of [^{3}H] CE in the liver was significantly lower in ITX5061-treated mice indicating that increased HDL-CE levels were due to reduced uptake by the liver (Fig 3E).

ITX5061 does not increase HDL levels in *SR-BI*$^{-/-}$ mice and reduces selective uptake via SR-BI in cell culture

Since SR-BI has a major role in the clearance of HDL-C and CE in the liver, we determined whether ITX5061 could still raise HDL levels in *SR-BI*$^{-/-}$ mice. WT and *SR-BI*$^{-/-}$ mice were treated for one week by daily administration of ITX5061 at 30mg/kg/day. While ITX5061 increased HDL-C in WT mice its effects were abolished in *SR-BI*$^{-/-}$ mice suggesting that the mechanism of action was dependent on SR-BI (Fig 4B). A significant increase in non HDL cholesterol levels was also observed in ITX5061-treated WT mice. Analysis of apolipoproteins by SDS-PAGE in fractions corresponding to the LDL region of similar profiles indicated they consisted primarily of apoB lipoproteins with only trace amounts of apoA-1 (data not shown). Interestingly despite increases in HDL-C, no increase in apoA-I was observed in ITX5061-treated WT mice (Fig 4A). We also confirmed by Western blot that ITX5061 did not affect the protein levels of apoA-I, ABCA1 and SR-BI in the liver (figure S4B)

To test the SR-BI inhibitory potential of ITX5061 *in vitro*, HEK 293 cells over expressing SR-BI were incubated with [^{3}H]CE labelled HDL. As shown in Fig 4C, ITX5061 significantly decreased HDL uptake at 1 μM concentration while a structurally related p38 MAPK inhibitor (KR-004515) had no effect at the same concentration.

ITX5061 increases HDL-C and reduces atherosclerosis in *Ldlr*^+/− mice fed the Paigen diet

Most prior studies of the effects of SR-BI deficiency or inhibition on atherogenesis have used *Ldlr*^−/− or *apoE*^−/− mice [7–9]. We wished to determine effects of ITX5061 on atherogenesis in a setting where the clearance of VLDL/LDL particles by apoE- or Ldlr-dependent pathways, is not completely impaired and with the potential availability of a compensatory pathway of reverse cholesterol transport via CETP. Indeed, it has been shown recently that CETP expression was able to reverse the atherogenic phenotype in *SR-BI*^−/− mice [10]. Thus, to determine the effects of ITX5061 on atherogenesis, *Ldlr*^+/− mice, with or without expression of CETP, were fed the Paigen diet containing ITX5061 (0.037 %) for 18 weeks. CETP expression was induced by injection of AAV-CETP [11] and was monitored two weeks later. Plasma CETP levels were slightly lower than obtained in a human plasma sample in mice injected with AAV-CETP but within the physiological range for humans (Fig S5). Plasma lipid parameters were monitored after 2 and 18 weeks of treatment. No differences in total cholesterol or TG levels were observed between ITX5061-treated or control mice. However, a 30 % increase in HDL-C concentrations was observed in ITX5061 treated groups as determined by FPLC (Fig 5A) and ultracentrifugation (Table SII). At human-like levels, CETP expression has only little effects on plasma lipid levels in WT mice as originally reported [12]. As expected, animals fed the Paigen diet developed moderately elevated transaminase levels [13] but there were no hyperbilirubinemia and there were no differences between the 4 groups (Table SII)

Following 18 weeks of ITX5061 administration, atherosclerotic lesion areas were first quantified by en face analysis of aortic arches. Data are reported as the percentage of the atherosclerotic lesions stained by Oil Red O on aortic surface. Fig 5B shows representative pictures of aortic arches from female mice with or without treatment by ITX5061. Lesions were observed primarily at branch points and sometimes along the lesser curvature. A significant 40% reduction of atherosclerotic lesions was observed in ITX5061-treated mice. Similar reductions in atherosclerosis were seen in ITX5061-treated mice injected with AAV-CETP or AAV-Ctrl (Fig 5C). No difference in lesion area was observed when comparing AAV-Ctrl groups and AAV-CETP groups either in control or ITX-5061 treated mice. Analysis of the lesions was also performed in the aortic valves (figure 5D). Although there was a trend towards reduced lesion area in both groups treated with ITX5061, the differences were not statistically significant.

Discussion

Our findings indicate that ITX5061, initially characterized as a p38 MAPK inhibitor, is able to increase HDL-C levels in humans and rodents and strongly suggest that this occurs at least in part through inhibition of SR-BI activity. Importantly, while previous studies have shown that modulation of SR-BI activity could affect apoB-containing lipoprotein levels, ITX5061 did not increase VLDL/LDL cholesterol or plasma TG concentrations in humans and *Ldlr*^+/− mice. Finally, although many studies have documented markedly increased atherosclerosis in various mouse models deficient in SR-BI, we observed that ITX5061 had no significant effect on atherosclerotic lesions in the aortic valves and reduced early atherosclerotic lesions in the aortic arch of *Ldlr*^+/− mice fed the Paigen diet for 18 weeks.

Several lines of evidence suggest that the primary mode of action of ITX5061 is via inhibition of SR-BI activity. First, similar to what is observed in *SR-BI*^−/− mice, ITX5061 increased HDL-C more than apoA-I and led to formation of large sized HDL enriched in cholesterol. Second, ITX5061 raised HDL-C by decreasing HDL-CE plasma catabolism and uptake by liver. Third, ITX5061 effects on HDL-C were abolished in *SR-BI*^−/− mice. Fourth, *in vitro*, ITX5061 inhibited SR-BI mediated HDL-CE uptake while other structurally related compounds without HDL raising ability did not. While ITX5061 was initially developed as a p38 MAPK inhibitor

it seems unlikely that this property contributed to increased HDL since structurally similar p38 MAPK inhibitors did not increase HDL levels. Nevertheless, the exact mechanism of SR-BI inhibition by ITX5061 remains to be determined. Other SR-BI inhibitors described to date decreased HDL-CE selective uptake and increased the binding of HDL to SR-BI suggesting the formation of inactive HDL-SR-BI complexes [14, 15].

The effects of SR-BI deficiency/inhibition on lipoprotein profiles are well documented in mice [5, 16, 17]. *SR-BI*^{−/−} mice display a marked increase in HDL-C concentration due to accumulation of very large size apoE- and cholesterol- and CE- enriched HDL. In the absence of apoE or Ldlr, SR-BI deficiency also increases VLDL/LDL concentration indicating a role for SR-BI as a back up receptor for these lipoproteins [16, 18−20]. In contrast to mice, much less is known about the effects of SR-BI deficiency or inhibition on lipoprotein profiles in humans. Several non functional SR-BI polymorphisms have been associated with changes in HDL and LDL cholesterol levels perhaps suggesting a role of SR-BI in humans [21−24]. No genetic deficiencies for SR-BI have been described to date [25, 26]. Only one preliminary report on administration of SR-BI inhibitory molecules in humans has been presented. HDL376, a molecule with SR-BI inhibitory properties was able to increase HDL-C levels in humans (+20 %) and also in non human primates [27, 28]. Our results suggest that the use of SR-BI inhibitors in humans has the potential to increase HDL-C and to a lesser extent apoA-I without detrimental effects on apoB-containing lipoprotein concentration, leading to a potentially less atherogenic lipoprotein profile. Interestingly, ITX5061 increased apoA-I as well as HDL-C in humans and in HuAITg mice, but in wild type mice only increased HDL-C levels. This suggests that there could be a difference in effects of SR-BI deficiency on HDL particles containing human apoA-I versus mouse apoA-I. Nevertheless, the increase of apo A-I was only moderate as compared to HDL-C, consistent with the SR-BI inhibition mechanism. The lack of increase VLDL and LDL cholesterol in humans treated with ITX5061 likely reflects the effect of partial SR-BI deficiency in a setting where apoE and Ldlr are still functional.

Following the discovery of dramatically increased HDL levels in *SR-BI*^{−/−} mice [5], there was considerable initial interest in the potential to use inhibition of SR-BI as an anti-atherogenic strategy. However, this idea was discarded in the face of compelling evidence that SR-BI deficiency increased atherosclerosis in several different mouse models, sometimes in a dramatic fashion, while SR-BI overexpression had the opposite effects [7−9, 16, 29−32]. Besides genetic models of SR-BI overexpression or deficiency, pharmacological SR-BI inhibitors such as BLTs, HDL376 and R-138329 have been characterized [14, 15, 27, 33, 34]. The effects of the latter compound on atherosclerosis have been investigated in *apoE*^{−/−} mice and moderate pro-atherogenic effects were observed but only at high concentration [35]. The increase in atherosclerosis seen in *SR-BI*^{−/−} mice has been attributed to the decrease in reverse cholesterol transport that has been clearly demonstrated in these mice [36]. In favor of this hypothesis, CETP expression was able to reverse the atherogenic phenotype in *SR-BI*^{−/−} mice[10]. However, in many of these studies [7, 8, 16] changes in HDL levels were associated with parallel changes in levels of VLDL and LDL, tending to confound the analysis of the mechanisms of atherogenesis. We did not find a pro-atherogenic effect of CETP on atherosclerosis in our model, either with or without ITX5061 treatment. This is possibly due to the low level of CETP expression at which we chose to work. CETP was previouly shown to be moderately pro-atherogenic in *Ldlr*^{−/−} that displayed much higher plasma CETP levels[37].

In the present study we observed that ITX5061 treatment resulted in a 40 % reduction in atherosclerosis in the aortic arch after 18 weeks of treatment in *Ldlr*^{+/−} mice fed the Paigen diet. The antiatherogenic effect of the molecule was observed in the control and in the CETP expressing group. Our results suggest therefore that partial SR-BI inhibition could have some beneficial effects *per se* whether an alternative pathway for HDL cholesterol uptake or transfer is provided by CETP or not.

Several points might account for differences between our results and those from previous studies. First we used a model of *Ldlr*^{+/−} mice where the clearance of apoB lipoproteins is not completely impaired leading only to moderate hypercholesterolemia. Secondly, SR-BI inhibition was not complete in our experimental conditions, only a 30–40 % increase in HDL levels was observed as compared with typical +125 % increases in *SR-BI*^{−/−} animals. Since it has been shown that SR-BI deficiency has different effects in liver and in vascular wall, selective inhibition of SR-BI by ITX5061 in different tissues could also explain some of the differences[38]. Third, while most studies used Western-type diet, we used a cholic acid containing diet, which have different effects on atherogenesis. Although this diet has been extensively employed in atherosclerosis studies [39], it is also recognized to have significant limitations. Indeed, cholate that is present in the Paigen diet induces liver inflammation and fibrosis and these effects contribute to the peculiar atherogenicity of the diet [40]. These concerns limit the usefulness of this model in extrapolating results to other settings such as in humans. Nonetheless, in the same dietary model marked overexpression of SR-BI was anti-atherogenic in conjunction with both HDL and VLDL/LDL lowering. Fourth, since ITX5061 is also a potent p38 MAPK inhibitor, it is possible that this off-target effect contributes, at least partially, to the reduction of atherosclerotic lesions in ITX5061-treated mice. However recent data do not support a role for p38 MAPK in regulating atherosclerotic lesion size. A p38 MAPK inhibitor decreased inflammation in atherosclerotic plaques but did not affect lesion size in *apoE*^{−/−} mice infused with angiotensin II [41]. Similarly, p38 MAPK deficiency in macrophages increased necrotic cores in advanced lesions in Western-type diet-fed *apoE*^{−/−} mice but did not change lesion size [42]. Therefore, although it seems unlikely that p38 MAPK inhibition contributed significantly to the reduction of atherosclerosis in our model, we cannot rule this out. In conclusion, inhibition of hepatic SR-BI in a setting that leads to increases in HDL without concomitant increases in VLDL and LDL cholesterol levels could potentially have an anti-atherogenic effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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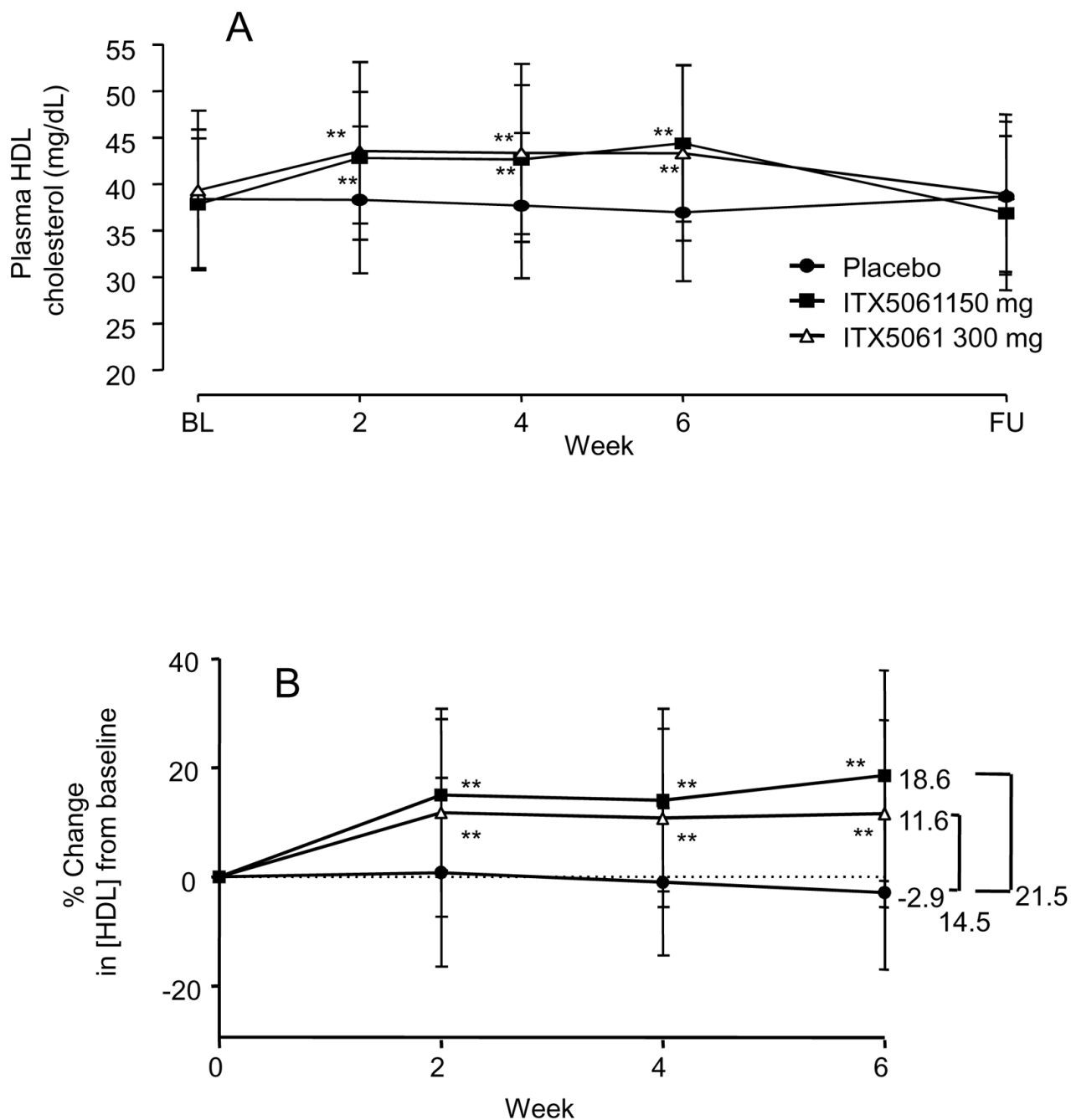
Figure 1. Changes in lipid parameters over time during ITX5061 administration
Patients received placebo (n=37), ITX5061 at 150mg (n=38) and ITX5061 at 300 mg (n=40) daily. Lipid parameters were determined at indicated time points. (A): Absolute change in HDL-C concentration. (B): Mean percent changes in HDL-C from baseline. Values are mean ± S.D. **P<0.05, ***P<0.01, P-values are against placebo, same time point, Kruskall-Wallis, Mann-Whitney test. .
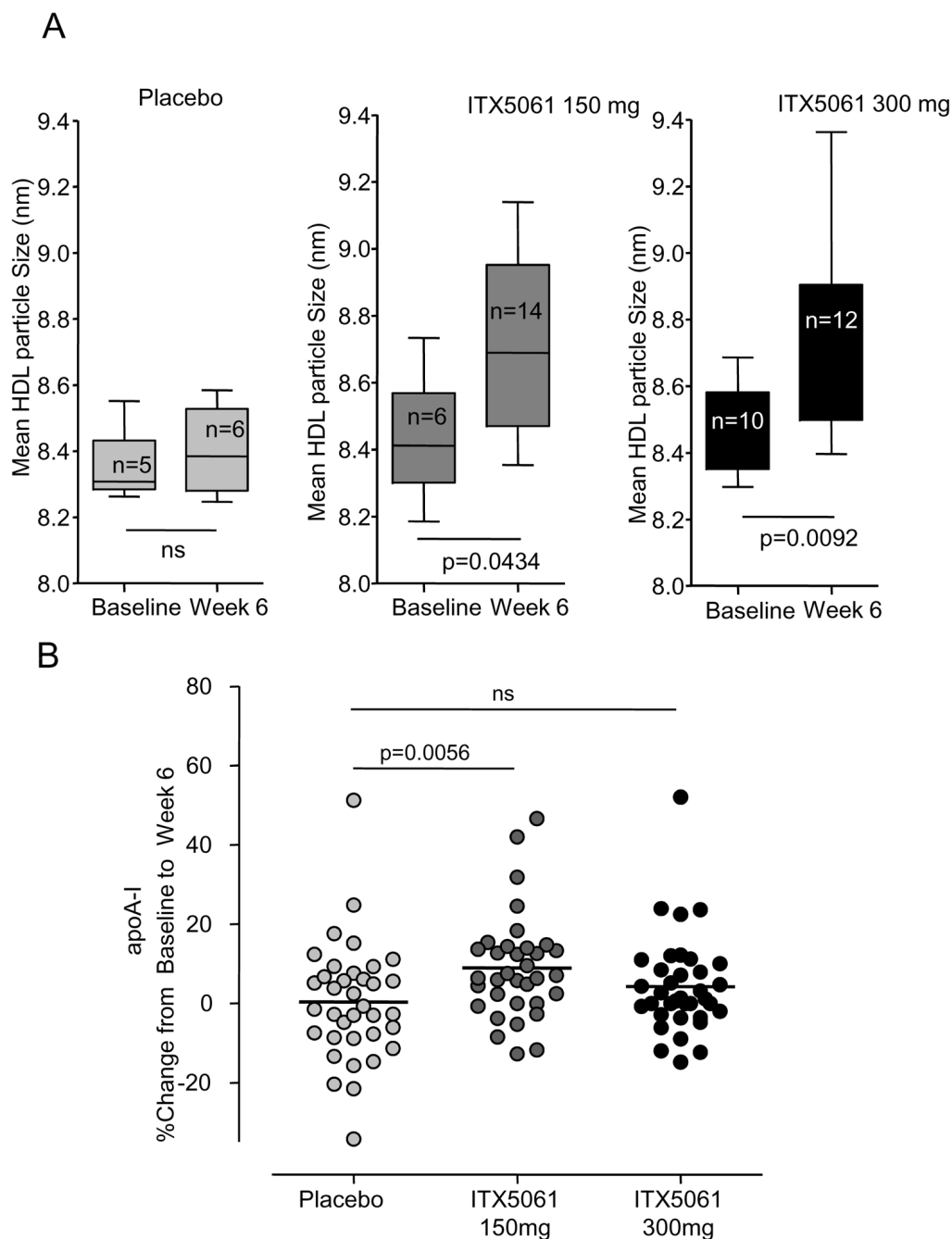
A

B

Figure 2.
(A) HDL size distribution in ITX5061 treated patients. HDL size was determined after 6 weeks of treatment using NMR. P-value based on Mann Whitney test. (B) **Effect of ITX5061 treatment on apoA-I concentration.** ApoA-I concentration was determined at the indicated time point. P-value based on Mann-Whitney test.
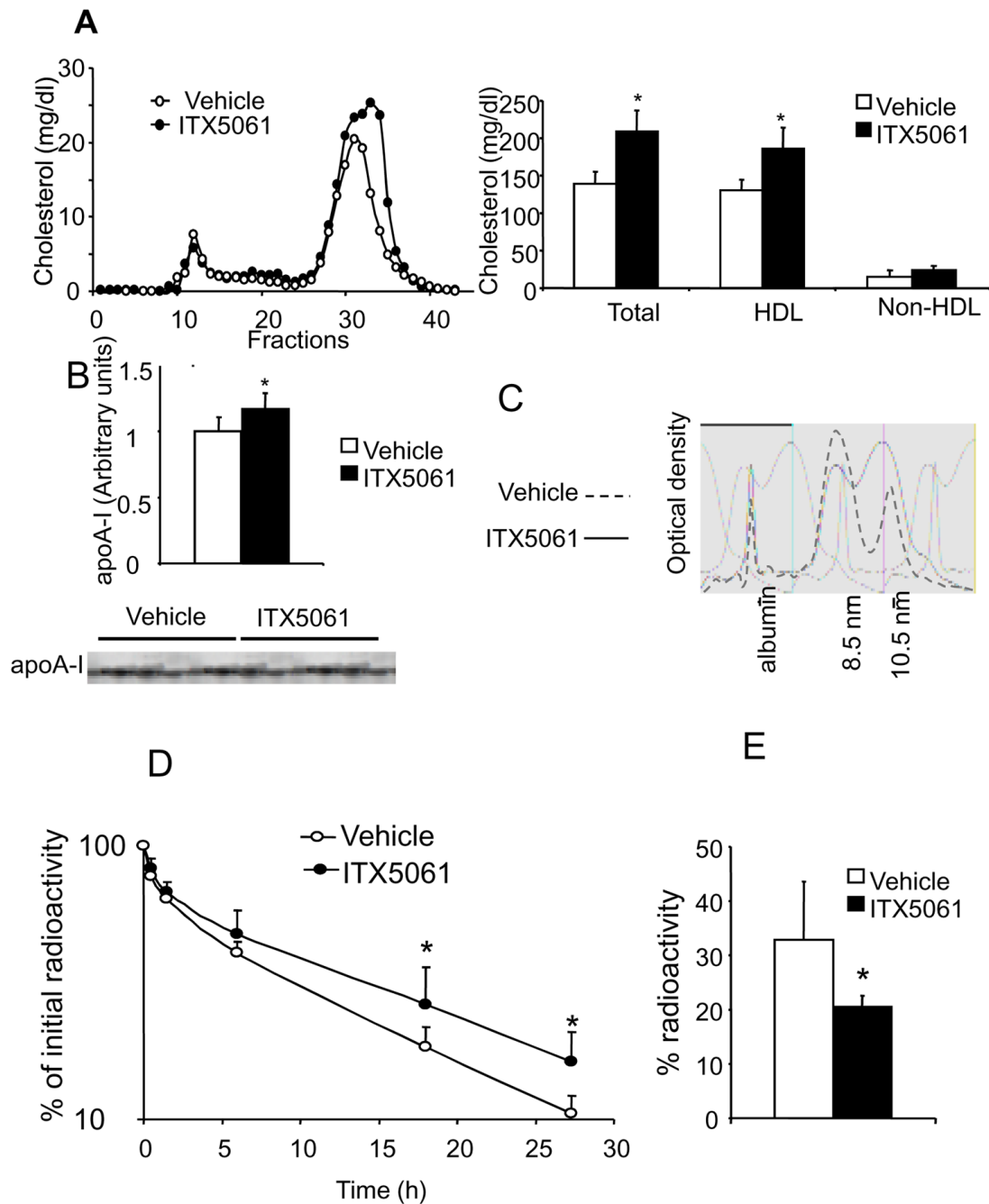
Figure 3.
(A) Plasma lipid levels and cholesterol distribution in HuAITg mice with or without ITX5061. HuAITg mice were treated for one week with ITX5061 or vehicle at 30 mg/kg/day. 100 μl of pooled plasma from 5 distinct mouse were used for FPLC analysis. Plasma HDL-C was determined after ultracentrifugation. * indicates a significant difference from mice receiving vehicle. (P<0.05 in both cases; Mann-Whitney test). **(B) apoAI concentrations in HuAITg mice with or without ITX5061**. * indicates a significant difference from mice receiving vehicle. (P<0.05; Mann-Whitney test). **(C) HDL size distribution in HuAITg mice with or without ITX5061.** HDL size was determined by native-PAGE. Profiles were determined in individual plasma samples. One presentative profile in each group is shown.

(D) Plasma kinetic of HDL labeled with [³H]CE in HuAITg mice with or without ITX5061. Values are the fraction of the injected dose remaining at each time point. The curves were fitted using a biexponential equation. Data are given as mean ± S.D.; n = 4 mice/group for each point. *: P<0.05 Vs vehicle, same time point, Mann-Whitney test. **(E) HDL-C uptake.** Values are expressed as the percentage of total injected radioactivity retrieved in the liver. *: P<0.05 Vs vehicle, Mann-Whitney test.
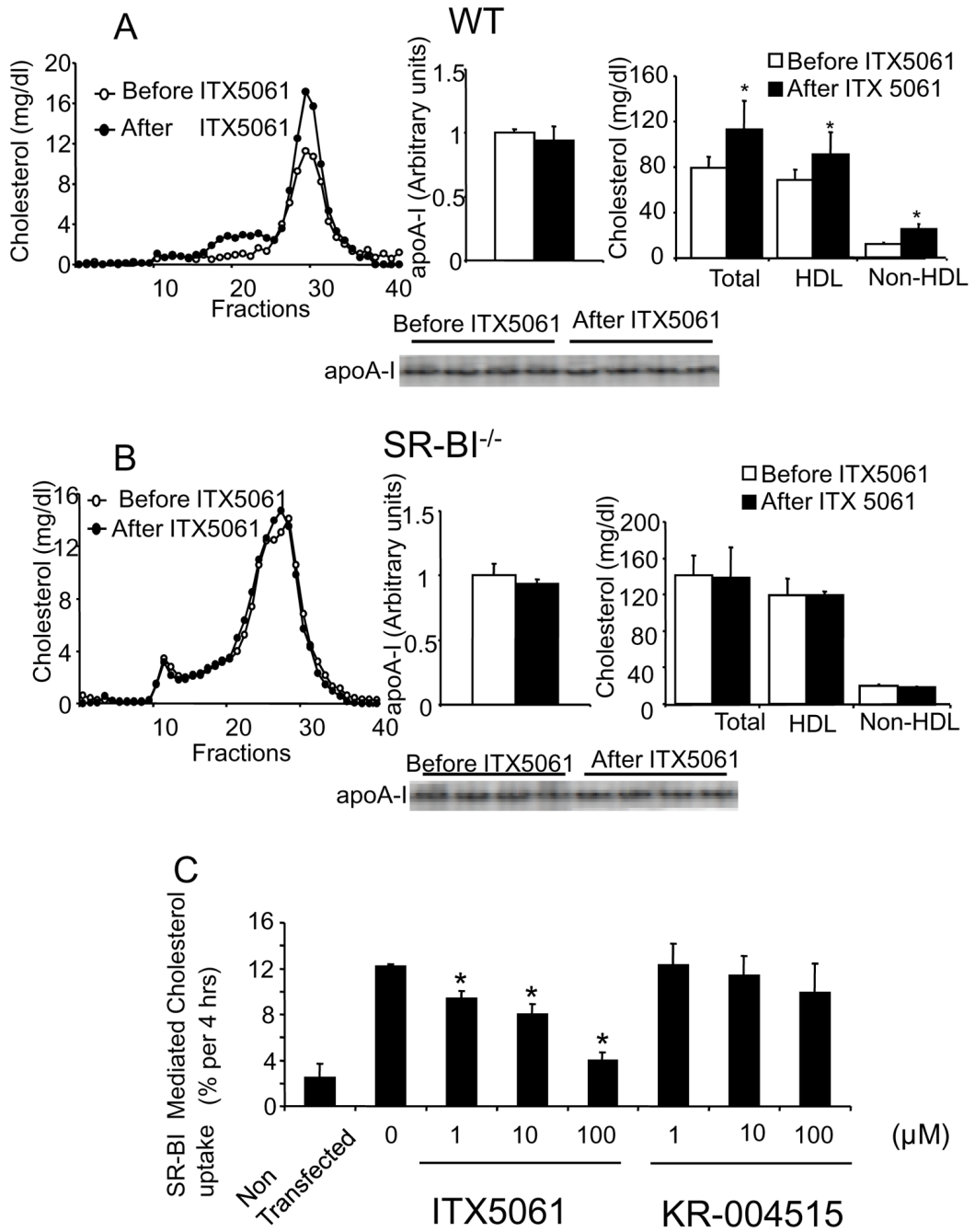
Figure 4. Plasma lipids, apoA-I concentrations and cholesterol distribution in WT and *SR-BI*^{−/−} mice with or without ITX5061

WT (A) and *SR-BI*^{−/−} mice (B) were treated for one week with ITX5061 or vehicle at 30 mg/kg/d. 100 μl of a pooled plasma from 4 distinct mouse were used for FPLC analysis. * indicates a significant difference from mice before treatment (P<0.05 in both cases; Wilcoxon test). **(C) Effect of ITX5061 on SR-BI mediated cholesterol uptake.** HEK 293 cells were transfected with SR-BI cDNA or a mock vector and HDL-C uptake was determined as describe in materials and methods (*: P<0.05 Vs 0 μM; Mann-Whitney test). Values obtained at 4°C were subtracted.
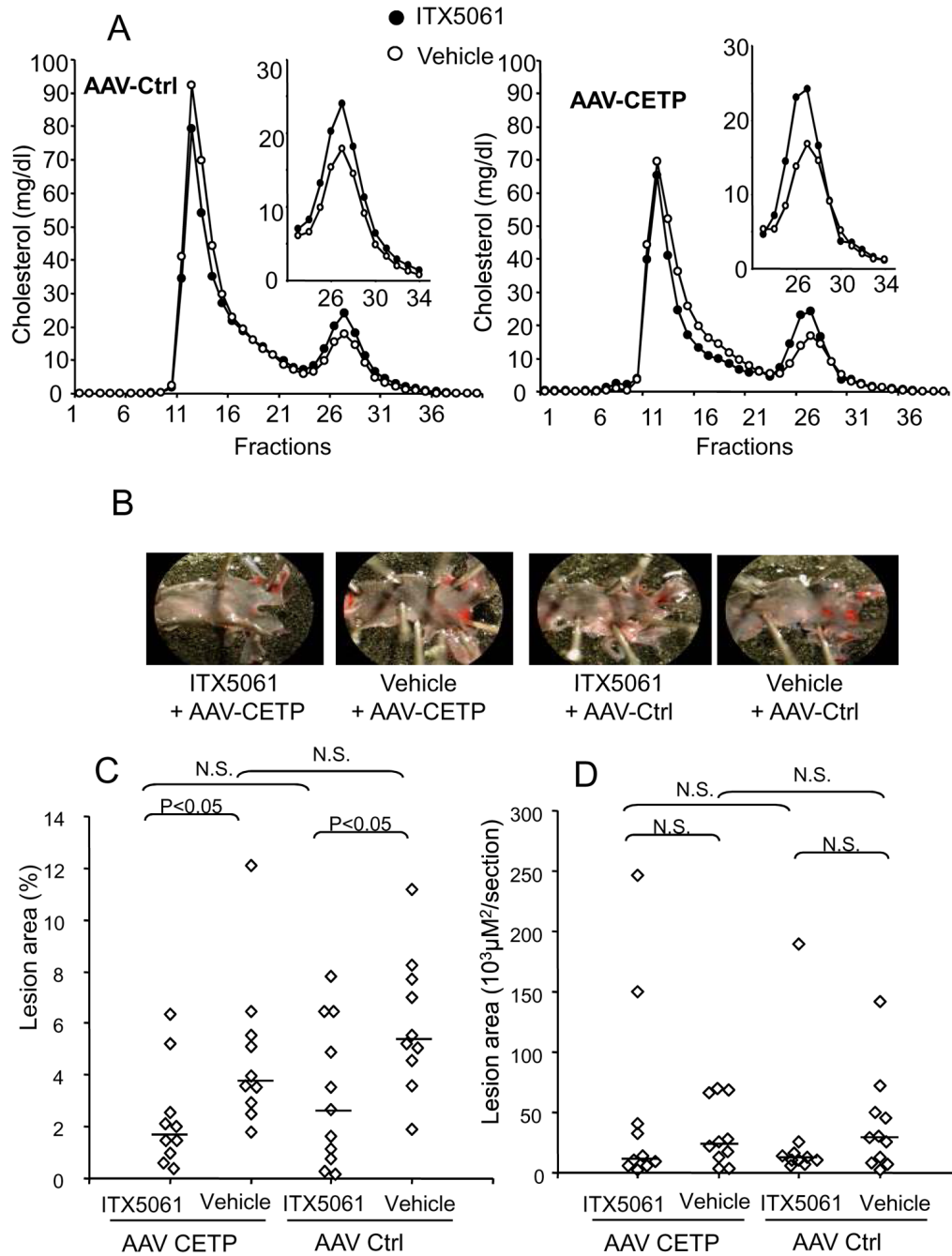
Figure 5.
(A) FPLC analysis of plasma cholesterol in male *Ldlr*^{+/−} mice with or without ITX5061. 100 μl of pooled plasma were used for FPLC analysis. **(B) Representative Oil Red O –stained aortas *Ldlr*^{+/−}** mice were fed the Paigen diet containing or not ITX5061 for 18 weeks. Aortas were stained with Oil Red O. **(C) Quantification of atherosclerosis lesions in *Ldlr*^{+/−} mice.** Oil Red O-stained lesions were quantified and expressed as a percent of total aortic area. Horizontal bars indicate the group medians. Statistical analysis by Kruskall-Wallis-Mann Whitney test **(D) Atherosclerotic lesion development in the proximal aorta.** Quantification of proximal aortic root lesion area was performed by morphometric analysis of H&E stained

sections. Values represent the average of 5 sections per mouse. Horizontal bars indicate the group medians Statistical analysis by Kruskall-Wallis-Mann Whitney test