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Inhibitory effect of PCSK9 on Abca1 protein expression and cholesterol efflux in macrophages

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Abstract

Background and aims - Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) may have extra-hepatic effects on cholesterol homeostasis of vascular macrophages. In this study, we aimed to investigate PCSK9 role on the anti-atherogenic process of ATP Binding Cassette transporter A1 (Abca1)-mediated cholesterol efflux. Methods- Abca1 cholesterol efflux was evaluated by a radioisotopic technique in mouse peritoneal macrophages (MPM) from wild-type (WT) or LDL receptor knock-out (*Ldlr*^{-/-}) mice exposed to human recombinant PCSK9, in presence of liver X receptor/retinoid X receptor (LXR/RXR) ligands or acetylated LDL (AcLDL) to stimulate Abca1 expression. Protein and gene expression were evaluated by western blot and quantitative real time PCR respectively. Results - PCSK9 inhibited Abca1-mediated cholesterol efflux induced by LXR/RXR agonists in WT MPM (-55%, p<0,0001) but not in *Ldlr*^{-/-} MPM. This effect was fully abrogated by the co-incubation with an anti-PCSK9 antibody. The inhibition of Abca1-dependent efflux induced by PCSK9 was associated with a reduction of Abca1 protein expression only in WT cells. *Abca1* gene expression was significantly downregulated by PCSK9 in WT macrophages (-64%, p<0.001) and, to a lesser extent, in MPM lacking *Ldlr* (-35%, p<0.001). The inhibitory effect on Abca1-mediated efflux was also confirmed in AcLDL-treated macrophages. PCSK9 had a marginal or no effect on the expression of the lipid transporters *Sr-b1* and *Abcg1*. Conclusions - PCSK9 plays a direct role on Abca1-mediated cholesterol efflux through a downregulation of *Abca1* gene and *Abca1* protein expression. This extrahepatic effect may influence relevant steps in the pathogenesis of atherosclerosis, such as foam cell formation.

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2 **Inhibitory effect of PCSK9 on Abca1 protein expression and cholesterol efflux in**
3 **macrophages**

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1. Introduction

Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) is a subtilisin family-serine protease mainly expressed in the liver ¹. It is generally accepted that the best characterized function of PCSK9 is the binding to hepatic LDL receptor (LDLr), leading to their degradation ². This process may occur both at intracellular level, where newly synthesized PCSK9 binds to LDLr in the Golgi system, thus preventing its transport to the cell membrane ³, and at extracellular level. In the latter case, PCSK9 secreted from hepatocytes binds to the epidermal growth factor repeat A region of LDLr, forming a complex that undergoes internalization and transport to the endosome/lysosome compartment ⁴. The following disruption of LDLr recycling leads to degradation of LDLr and the consequent reduction of receptors number on the cell surface. Both these mechanisms largely contribute to regulate plasma LDL cholesterol levels and account for the identification of PCSK9 as a promising pharmacological target to manage hypercholesterolemia ⁵.

Although the above described activity appears to be the primary mechanism of action of PCSK9, relevant extrahepatic effects affecting cholesterol homeostasis have been also described. For instance, PCSK9 regulates the expression and activity of proteins involved in lipid transport and lipoprotein assembly in the intestine ^{6,7} and is an important player in the trans-intestinal cholesterol efflux ⁸.

Recent studies have documented PCSK9 role in different processes involved in the development of atherosclerosis, independently on the regulation of circulating LDL cholesterol levels. Our group demonstrated that PCSK9 is secreted by smooth muscle cells of human atherosclerotic plaques and acts, in a paracrine manner, on vessel macrophages by reducing LDLr expression and LDL uptake ⁹. **In addition, PCSK9 demonstrated a significant impact on restenosis in an experimental model of vascular injury** ¹⁰. Tang and colleagues showed that PCSK9 silencing in THP-1 macrophages results in attenuation of foam cell formation through the down-regulation of CD36 expression ¹¹. Moreover, PCSK9 impact on inflammatory and on apoptotic processes involved in atherogenesis has been proposed by some authors ^{12,13, 14}.

1 Cholesterol efflux from macrophages is a well recognized antiatherogenic process, promoting the
2 removal of excess cholesterol and thus preventing the formation of foam cells in the atherosclerotic
3 plaque¹⁵. It occurs by different pathways, involving lipid transporters, such as ATP Binding
4 Cassette transporter A1 (ABCA1), ATP Binding Cassette transporter G1 (ABCG1) and Scavenger
5 Receptor Class B Type I (SR-BI), or aspecific mechanisms¹⁶.

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10 The potential impact of PCSK9 on cholesterol efflux has been already considered by Shen and
11 colleagues that speculated on a possible impairment of the main lipid transporters¹⁷. However, up
12 to now no direct evidence of PCSK9 ability to affect cholesterol efflux has been provided. The aim
13 of our study is to directly investigate the potential effects of PCSK9 on cholesterol efflux, especially
14 the mechanism mediated by Abca1, one of the most relevant lipid transporter in macrophages^{18,19}.

24 **2. Materials and Methods**

28 *2.1 Cells*

29 Mouse peritoneal macrophages (MPM) were collected from C57BL/6 wild type or Ldlr knock-out
30 (*Ldlr*^{-/-}) mice (The Jackson Laboratory, Bar Harbour, ME, USA) peritoneal cavity as previously
31 described¹⁶. Briefly, 5 days after the injection of mice with 3 ml of 4% thioglycollate (Sigma Aldrich
32 St. Louis, MO, USA), peritoneal macrophages were collected from the mouse abdomen by lavage
33 with a sucrose solution. Cells were cultured in RPMI 1640 (from Lonza Walkersville, MD, USA)
34 medium supplemented with 10% fetal calf serum (FCS) and gentamicin (Invitrogen, Eugene, OR)
35 and incubated at 37°C and 5% CO₂.

48 *2.2 Cell cholesterol efflux*

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52 Cells were seeded in 24-well plates for 24 h in medium supplemented with 10% FCS and
53 gentamicin. In the experiments using liver X receptor (LXR) and retinoic X receptor (RXR)
54 agonists, cells were labeled with 2 µCi/ml [1,2-³H]-cholesterol in RPMI 1640 + 2.5% FCS.

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59 Subsequently cells were incubated with or without human recombinant PCSK9 C-terminal His-tag

1 (BPS-71204, Bioscience Inc., San Diego, CA) at 6.4 µg/ml in RPMI 1640 in 0.2% bovine serum
2 albumin (BSA, Sigma Aldrich)-containing medium for 32 h. The concentration of human
3 recombinant PCSK9 has been selected based on previous work²⁰. Then, cells were treated for 18 h
4 with LXR/RXR agonists, 22 (R) hydroxycholesterol (22-OH) 5 µg/ml and 9-cis retinoic acid (9cRA)
5 10 µM (both from Sigma Aldrich), to induce Abca1 expression, again with or without PCSK9 (6.4
6 µg/ml). Where indicated, PCSK9 treatment was carried out in combination with 12 µg/ml of an anti-
7 PCSK9 antibody (Goat polyclonal Ab anti PCSK9, Abcam ab28770) or with 12 µg/ml of normal
8 isotype IgG (Sigma Aldrich), as a negative control. Cholesterol efflux was promoted for 4 h to
9 apolipoprotein A-I (apoA-I) in RPMI 1640. The ApoA-I used for efflux experiments was purchased
10 by Sigma Aldrich. As indicated by manufacturer's technical sheet, apoA-I was isolated as lipid free
11 from human plasma. The product is supplied as a frozen solution in 10 mM NH₄HCO₃, pH 7.4.
12 Purity: Minimum 85% (SDS-PAGE). The radioactivity present in the medium was determined by
13 liquid scintillation counting. Cell [³H]cholesterol content was extracted with 0.6 ml of 2-propanol.
14 The lipid extracts were dried under N₂, resuspended in toluene, and quantified by liquid scintillation
15 counting. Cholesterol efflux was expressed as counts per minute in medium/(counts per minute in
16 medium + counts per minute in monolayers) X 100. Cholesterol efflux from macrophage foam cells
17 was evaluated in macrophages radioabeled with 2 µCi/ml [1,2-³H]-cholesterol in RPMI 1640 +
18 2.5% FCS in presence of AcLDL 25ug/ml for 24h. Successively, PCSK9 6.4µg/ml was incubated
19 for 32 h + 18 h in a BSA-containing medium. Cholesterol efflux was promoted to apoA-I and
20 calculated as described above.
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45 *2.3 Western Blotting*

46 MPM were seeded in 6-wells plates in 10% FCS-containing medium for 24 and incubated with or
47 without human recombinant PCSK9 C-terminal His-tag (BPS-71204, Bioscience Inc., San Diego,
48 CA) at 6.4 µg/ml in a 0.2% BSA-containing medium for 32 h. Successively, Abca1 expression was
49 induced by treatment with LXR/RXR agonists, an association of 5 µg/ml 22-OH and 10 µM 9cRA
50 (both from Sigma Aldrich) for 18 h in 0.2 % BSA containing-RPMI again in the presence or
51 absence of human recombinant PCSK9 6.4 µg/ml. Cells were then washed twice with cold PBS
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1 and lysed using lysis buffer (Trizma 10 mM, MgCl₂ 1 mM, NP-40 0.5%, Triton x-100 1%)
2 containing a protease inhibitors cocktail (Sigma Aldrich). Cell lysate aliquots at fixed protein
3 content (30 µg), measured using the BCA protein assay (Pierce, Rockford, IL, USA)²¹, were
4 separated on 8% acrylamide gels and transferred to nitrocellulose membranes (Biorad, Hercules,
5 CA, USA). Abca1 specific -rabbit primary antibody (Novus Biologicals, Littleton, CO, USA) and an
6 anti-rabbit IgG horseradish peroxidase-conjugated (GE Healthcare Life Sciences,
7 Buckinghamshire, UK) were used to incubate the blots. An anti-actin antibody (Novus Biologicals,
8 Littleton, CO USA) was used to visualize actin as a control protein. Antibody binding visualization
9 was obtained by enhanced chemiluminescence (Pierce™ ECL Plus, Thermo Fisher Scientific,
10 MA, USA), according to the manufacturer's instructions. Images were analyzed using Image
11 Studio™ software by two independent blind observers.
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26 *2.4 RNA preparation and quantitative real time PCR*

27 MPM were seeded in 6-wells plates in 10% FCS-containing medium for 24h and treated as
28 described above for cholesterol efflux assay. Total RNA was extracted with Nucleo Spin RNA Plus
29 kit (Carlo Erba Reagents, Milan, Italy) according to manufacturer's instructions. Reverse
30 transcription-polymerase first-strand cDNA synthesis was performed by using the iScript cDNA
31 synthesis Kit (BIO-RAD laboratories). Quantitative real time PCR (qPCR) was then performed by
32 using the Kit Thermo SYBR Green/ROX qPCR Master Mix (Carlo Erba Reagents, Milan, Italy) and
33 specific primers for selected genes. The primers used for qPCR are listed below. Primers used for
34 mRNA analysis are mouse *Abca1* forward, 5'-GGT TTG GAG ATG GTT ATA CAA TAG TTG T-3';
35 *Abca1* reverse, 5'-GGT TTG GAG ATG GTT ATA CAA TAG TTG T-3'; mouse *Abcg1* forward, 5'-
36 TTC CCG GAA ACG CAA GTC-3'; *Abcg1* reverse, 5'-CAG TAG GCC ACA GGG AAC AT-3'; *Sr-bi*
37 forward, 5'-GGC TGC TGT TTG CTG CG-3'; *Sr-bi* reverse 5'-GCT GCT TGA TGA GGG AGG G-
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55 The analyses were performed with the ABI Prism® 7000 Sequence Detection System (Applied
56 Biosystems; Life Technologies Europe BV, Milan, Italy). PCR cycling conditions were as follows:
57 94°C for 3min, 40 cycles at 94°C for 15s, and 60°C for 1min. Data were expressed as Ct values
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1 and used for the relative quantification of targets with the $\Delta\Delta C_t$ calculation, using 18S as
2 housekeeping gene²².

3 2.5 Statistical analyses

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8 Each experimental condition was run in triplicate and data were expressed as mean \pm SD, as
9 representative of three or more experiments. Statistical analyses were performed using Prism 6.0
10 (GraphPad Inc., San Diego, CA, USA). Multiple comparisons among more than two means were
11 performed by one way ANOVA with a post hoc Tukey correction. Significant differences were
12 defined as $p < 0.05$.
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22 3. Results

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24 To explore a potential effect of PCSK9 on Abca1-mediated cholesterol efflux we first used MPM
25 stimulated with the nuclear receptors LXR/RXR ligands 22-OH and 9cRA in order to upregulate
26 Abca1 expression¹⁵. As expected, treatment with LXR/RXR agonists determined an increase of
27 about 2.5-fold of cholesterol efflux to apoA-I (ANOVA, $p < 0.001$). Co-incubation of cells with human
28 recombinant PCSK9 inhibited this effect by 55% (ANOVA $p < 0.05$). Conversely, PCSK9 did not
29 affect cholesterol efflux to apoA-I under basal conditions (Figure 1, panel A).
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37 To investigate the involvement of Ldlr in the PCSK9-induced inhibitory effect on Abca1-mediated
38 cholesterol efflux, we conducted the same experiment in *Ldlr*^{-/-} MPM. Lxr/Rxr activation determined
39 a significant induction of Abca1-mediated cholesterol efflux (from 0.61% \pm 0.02 to 1.02% \pm 0.03,
40 ANOVA $p < 0.05$) despite to a lower extent compared to WT cells. Differently from what observed in
41 WT cells, PCSK9 failed to inhibit cholesterol efflux in MPM lacking *Ldlr* (Figure 1, panel B). In the
42 absence of Lxr/Rxr-induction of Abca1 expression, PCSK9 did not influence cholesterol efflux in
43 *Ldlr*^{-/-} macrophages (Figure 1, panel B).
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53 To provide evidence for the specificity of the inhibitory effect of PCSK9 on Abca1-mediated
54 cholesterol efflux, under the same experimental conditions described above, we treated WT
55 macrophages with PCSK9 alone or in combination with an anti-PCSK9 antibody. As shown in
56 Figure 2, while PCSK9 completely abrogated Abca1-dependent cholesterol efflux (ANOVA
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p<0.0001), co-incubation of PCSK9 with the anti-PCSK9 antibody completely prevented this effect.

To further demonstrate the specificity of this effect, a set of cells was treated with normal isotype IgG in the absence or presence of human recombinant PCSK9. In the latter condition PCSK9 inhibitory effect was fully preserved (Figure 2).

To investigate whether the observed effect on cholesterol efflux was a consequence of a modulation of Abca1 protein expression by PCSK9, we performed a western blot analysis in the same experimental conditions as above. Cell treatment with LXR/RXR agonists significantly up regulated the expression of the cholesterol transporter Abca1 in both WT and *Ldlr*^{-/-} macrophages (Figure 3, panels A and B). The addition of PCSK9 significantly inhibited the protein expression in WT (Figure 3, panel A), but not in *Ldlr*^{-/-} MPM (Figure 3, panel B).

In a parallel set of cells, we also evaluated the impact of human recombinant PCSK9 on *Abca1* gene expression by quantitative real time PCR. LXR/RXR agonists significantly up regulated *Abca1* expression (ANOVA, p<0.0001) both in WT and *Ldlr*^{-/-} macrophages. Treatment with PCSK9 resulted in a 64% and 35% reduction of *Abca1* mRNA (ANOVA, p<0,001) in WT and *Ldlr*^{-/-} macrophages respectively (Figure 4, panel A and B). PCSK9 did not affect the basal expression of *Abca1* in both WT and *Ldlr*^{-/-} macrophages (Figure 4, panel A and B respectively).

To address the question if PCSK9 similarly regulates the expression of other LXR/RXR target genes, we evaluated PCSK9 effect on *Abcg1* gene in WT macrophages. As shown in Figure 5, panel A, no significant effect was detected. Finally, we evaluated the influence of PCSK9 on *Sr-bi* gene, that encodes for another cholesterol transporter involved in cholesterol efflux. PCSK9 caused a slight, but statistically significant, down regulation of *Sr-bi* (ANOVA, p<0.05) (Figure 5, panel B).

We finally assessed whether the observed effects of PCSK9 were confirmed in a model of foam cells. Treatment of macrophages with AcLDL increased cholesterol efflux to apoA-I from 0.81% ± 0.03 to 1.08% ± 0.17 (ANOVA, p<0.05). Co-incubation with PCSK9 fully inhibited the cholesterol loading-induced Abca1-mediated cholesterol efflux (from 1.08% ± 0.17 to 0.85% ± 0.03; ANOVA, p<0.05). When we evaluated the mRNA levels of the cholesterol transporters *Abca1*, *Abcg1* and *Sr-bi* in this experimental condition, we did not detect any effect of PCSK9 (data not shown).

4. Discussion

In this work, for the first time, we provide evidence that PCSK9 affects macrophage cholesterol homeostasis by inhibiting the process of Abca1-mediated cholesterol efflux induced by the activation of LXR/RXR pathway. The specificity of this effect was demonstrated by the loss of PCSK9 activity in presence of an anti-PCSK9 antibody. Conversely, when macrophages were incubated with a normal isotype IgG, PCSK9 inhibition was preserved. We further show that in cells treated with LXR/RXR agonists the inhibitory effect of PCSK9 on Abca1-mediated cholesterol efflux was associated to a significant reduction of both Abca1 protein expression and *Abca1* mRNA content. In *Ldlr*^{-/-} macrophages, PCSK9 did not affect neither Abca1-mediated efflux nor Abca1 expression induced by Lxr/Rxr stimulation. These results indicate that PCSK9 inhibited the Abca1-dependent cholesterol efflux by affecting its protein expression and this effect required the presence of the LDLr.

Surprisingly, in *Ldlr*^{-/-} macrophages we observed a differential effect of PCSK9 on Abca1 protein/function and *Abca1* mRNA levels, since PCSK9 was still capable to prevent *Abca1* mRNA expression, although to a lower extent as compared to wild type macrophages. This residual effect may depend on PCSK9 internalization with other lipoprotein receptors, such as LDLr related protein-1, the receptor for VLDL, apoER2^{23,24,25} or the scavenger receptor CD36 expressed in macrophages¹¹.

We observed a discrepancy between PCSK9 impact on Abca1 function and gene expression also in the experimental model of foam cells: whereas the former is significantly inhibited, the latter does not seem to be affected. This observation suggest a post-transcriptional regulation by PCSK9 that still needs to be elucidated.

The requirement of the LDLr for PCSK9 extra-hepatic effects, in particular at the level of macrophages, was recently documented by Fazio et al¹². The authors showed that PCSK9 facilitates the macrophage response to LPS by increasing the expression of pro-inflammatory cytokines interleukin1- β and tumor necrosis factor- α and reducing the anti-inflammatory

1 interleukin10 and arginase-1 in a LDLr-dependent manner ¹². In addition, the authors reported that
2 macrophages express detectable levels of PCSK9. This result is in contrast with our previous
3 observation and with data reported by others revealing that MPM secrete null or very low amount
4 of endogenous PCSK9 ^{3,9}. We showed here that the incubation of macrophages with an anti-
5 PCSK9 antibody caused a slight increase of cholesterol efflux, as compared to control cells, at the
6 same extent observed upon incubation with normal isotype IgG. All together these observations
7 make unlikely the involvement of endogenous PCSK9 on cholesterol efflux in our experimental
8 conditions.
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10 The exact mechanism by which PCSK9 inhibits Lxr/Rxr-induced cholesterol efflux remains to be
11 established. A direct effect on Lxr/Rxr expression or activity is not supported by the observation
12 that PCSK9 did not affect the Lxr/Rxr-dependent *Abcg1* expression.
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14 Our present study adds an extra layer of complexity on the role of PCSK9 in atherogenesis and
15 macrophage functions. Cholesterol efflux to extracellular acceptors is the only process by which
16 macrophages protect themselves from the toxic effects of excess intracellular free cholesterol ²⁶.
17 Thus, the inhibition of this mechanism may favour foam cell formation, an hallmark of
18 atherosclerosis. Cell cholesterol efflux mainly occurs through the activity of the membrane
19 transporters ABCA1, ABCG1 and SR-BI in presence of extracellular cholesterol acceptors such as
20 lipid free apolipoprotein A-I and HDL ¹⁵. The ABCA1-mediated process in particular, has a pivotal
21 role in the trafficking of lipids and overall cholesterol homeostasis as demonstrated by the
22 increased atherosclerosis in hyperlipidemic mice with inactivation of *Abca1* in macrophages²⁷.
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24 In this study we also reported the impact of PCSK9 on other main genes involved in
25 cholesterol efflux: *Abcg1* ²⁸ and *Sr-bi* ²⁹. Differently from *Abca1*, *Abcg1* mRNA was not significantly
26 affected by PCSK9 in cells treated with LXR/RXR ligands. On the contrary, PCSK9 caused a slight
27 but statistically significant down regulation of *Sr-bi* expression. However, given the negligible
28 contribution of this receptor to overall cholesterol efflux from macrophages ¹⁶ it is likely that the
29 impact of PCSK9 on Sr-Bi-mediated cholesterol efflux would not be relevant.
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31 In conclusion, our study provides new insights into PCSK9 pro-atherogenic action, showing
32 a new mechanism by which PCSK9 directly dysregulates cholesterol homeostasis in macrophages
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as it inhibits cholesterol efflux mediated by Abca1. This novel observation could implement our knowledge on the physiological role of PCSK9 and opens to new potential anti-atherosclerotic properties of PCSK9 inhibition, independently of the regulation of LDL cholesterol levels.

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Conflict of interest

None.

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Author contributions

N.F. and A.C. conceived the core study and M.P.A., I.Z., N.F. and E.F. developed it. M.P.A., I.Z. (corresponding author), E.C. C.R., F.Z., performed the experiments. N.F., M.P.A., I.Z. (corresponding author), F.B. and A.C. analyzed and interpret the data; M.P.A., I.Z. (corresponding author), N.F. have written the manuscript. All authors have revised the manuscript critically for important intellectual content, and have given final approval of the version to be submitted.

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Figure captions

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4 **Figure 1. Effect of PCSK9 on Abca1-mediated cholesterol efflux from WT and from *Ldlr*^{-/-}**
5 **MPM.** WT MPM (panel A) and *Ldlr*^{-/-} MPM (panel B) were radiolabeled and subsequently incubated
6
7 in the absence or presence of human recombinant PCSK9 and of LXR/RXR agonists as described
8
9 in the Methods section. Cholesterol efflux was promoted to apoA-I for 4 h. Each cell treatment was
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11 performed in triplicate and data are expressed as mean ± SD. Shown data are representative of at
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13 least three separate experiments. One way ANOVA with a post hoc Tukey correction was run to
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15 compare the different means. A value of $p < 0.05$ was considered statistically significant. *= $p < 0.05$;
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17 ***= $p < 0.001$.

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26 **Figure 2. Effect of anti-PCSK9 antibody on Abca1-mediated cholesterol efflux from WT**
27 **MPM.** MPM were radiolabeled and subsequently incubated in the absence or presence of human
28
29 recombinant PCSK9 and LXR/RXR agonists as described in the Methods section. To assess the
30
31 specificity of PCSK9 effect, an antibody anti-PCSK9 or human IgG were added to cells at the same
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33 incubation time as PCSK9. Cholesterol efflux was promoted to apoA-I for 4 h. Each cell treatment
34
35 was performed in triplicate and data are expressed as mean ± SD. Shown data are representative
36
37 of at least three separate experiments. One way ANOVA with a post hoc Tukey correction was run
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39 to compare the different means. A value of $p < 0.05$ was considered statistically significant.
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41 ***= $p < 0.001$; ****= $p < 0.0001$

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50 **Figure 3. Effect of PCSK9 on Abca1 protein expression in WT and in *Ldlr*^{-/-} MPM.** WT MPM
51
52 (panel A) and *Ldlr*^{-/-} MPM (panel B) were incubated in the absence or presence of human
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54 recombinant PCSK9 and of LXR/RXR agonists as described in the Methods section. At the end of
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56 treatments, protein expression analysis was performed by western blotting and reported on the left
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58 of each panel. Signal quantification, measured with Image Studio™ software, is reported on the
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1 right of each panel. Data are expressed as means \pm SD. One way ANOVA with a post hoc Tukey
2 correction was run to compare the different means. A value of $p<0.05$ was considered statistically
3 significant; ***= $p<0,001$; ****= $p<0,0001$.
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10 **Figure 4. Effect of PCSK9 on *Abca1* gene expression in WT and in *Ldlr*^{-/-} MPM.** WT MPM
11 (panel A) and *Ldlr*^{-/-} MPM (panel B) were incubated in the absence or presence of human
12 recombinant PCSK9 and of LXR/RXR agonists as described in the Methods section. *Abca1* mRNA
13 levels were determined by quantitative RT-PCR. Each cell treatment was performed in triplicate
14 and data are expressed as means \pm SD. Shown data are representative of at least three separate
15 experiments. One way ANOVA with a post hoc Tukey correction was run to compare the different
16 means. A value of $p<0.05$ was considered statistically significant; ***= $p<0,001$; ****= $p<0;0001$.
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30 **Figure 5. Effect of PCSK9 on *Abcg1* and *Sr-bi* gene expression in WT macrophages.** MPM
31 were incubated in the absence or presence of human recombinant PCSK9 and of LXR/RXR
32 agonists as described in the Methods section. *Abcg1* (panel A) and *Sr-bi* (panel B) mRNA levels
33 were determined by quantitative RT-PCR. Each cell treatment was performed in triplicate and data
34 are expressed as means \pm SD. Shown data are representative of at least three separate
35 experiments. One way ANOVA with a post hoc Tukey correction was run to compare the different
36 means. A value of $p<0.05$ was considered statistically significant; *= $p<0,05$.
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Figure 1
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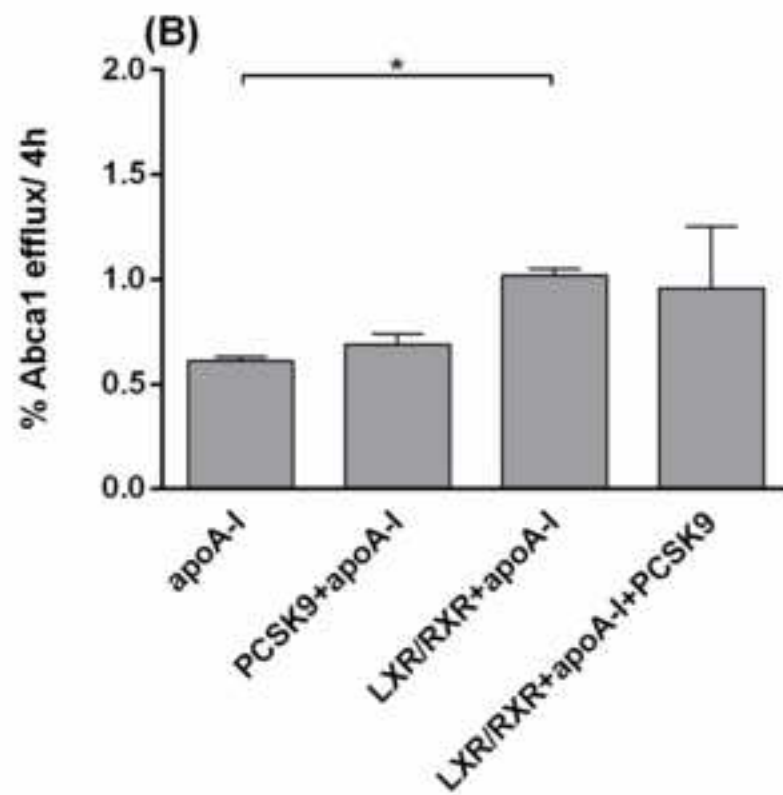
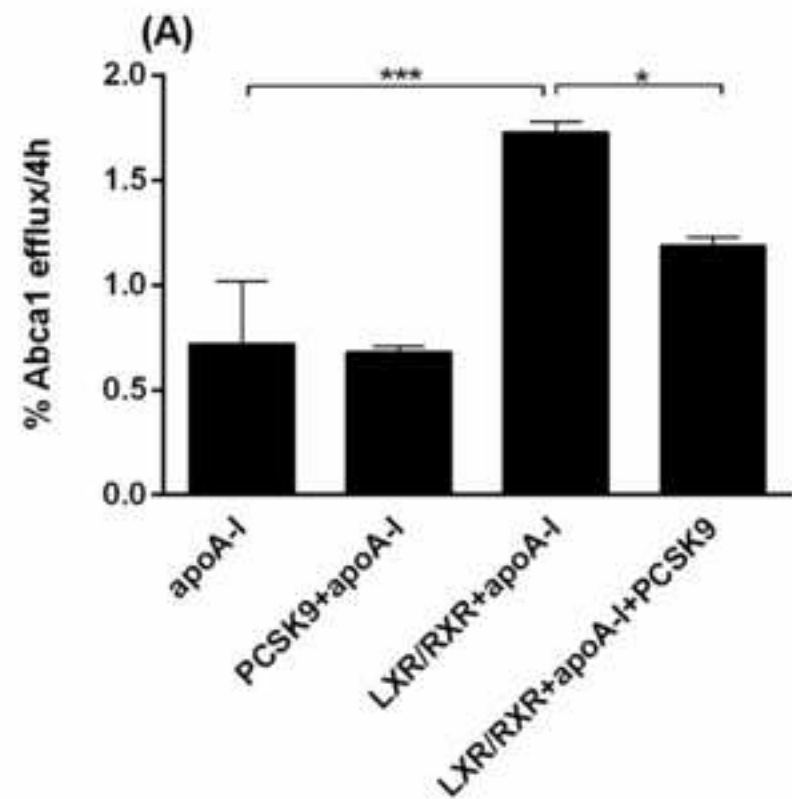


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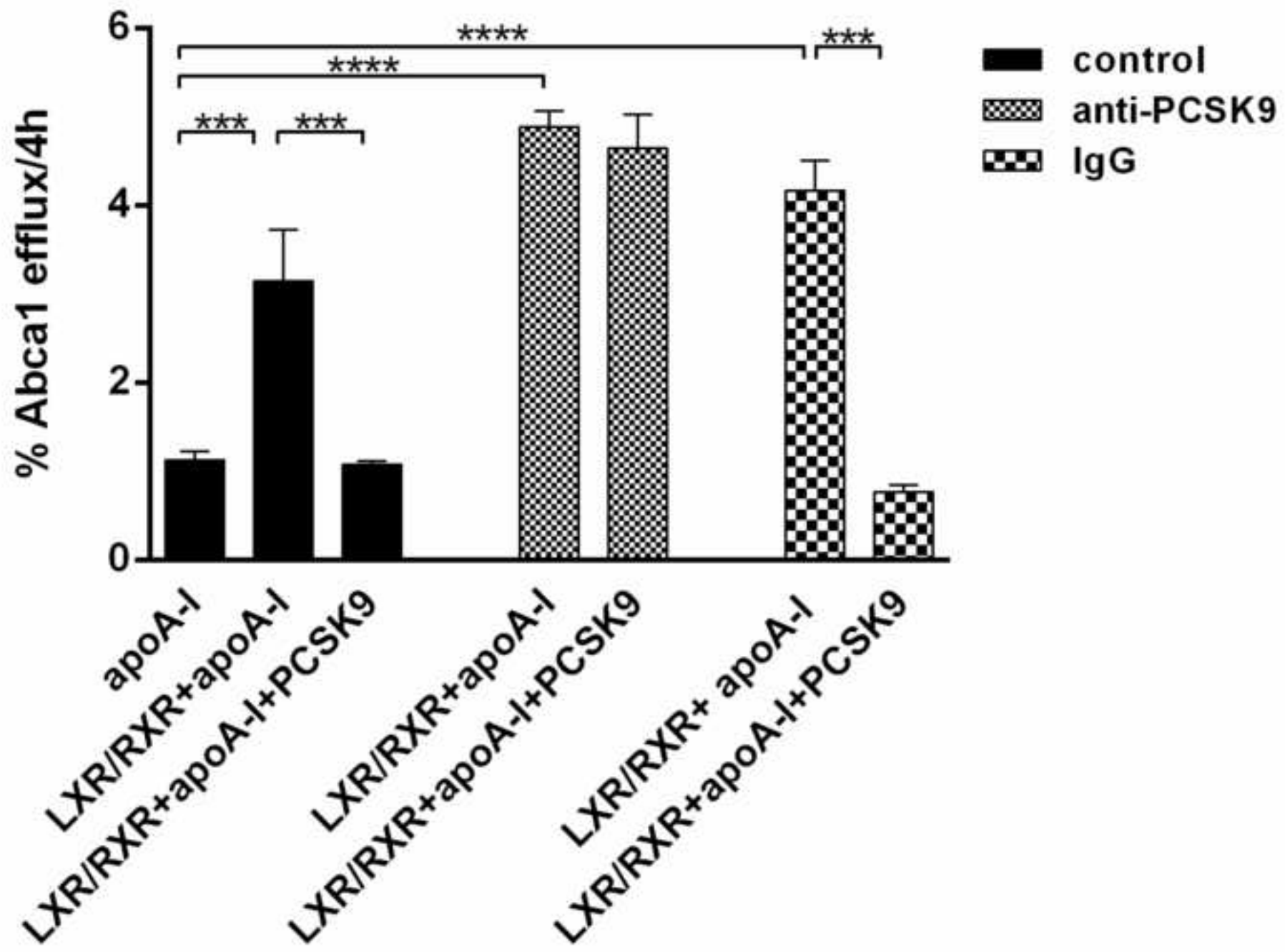


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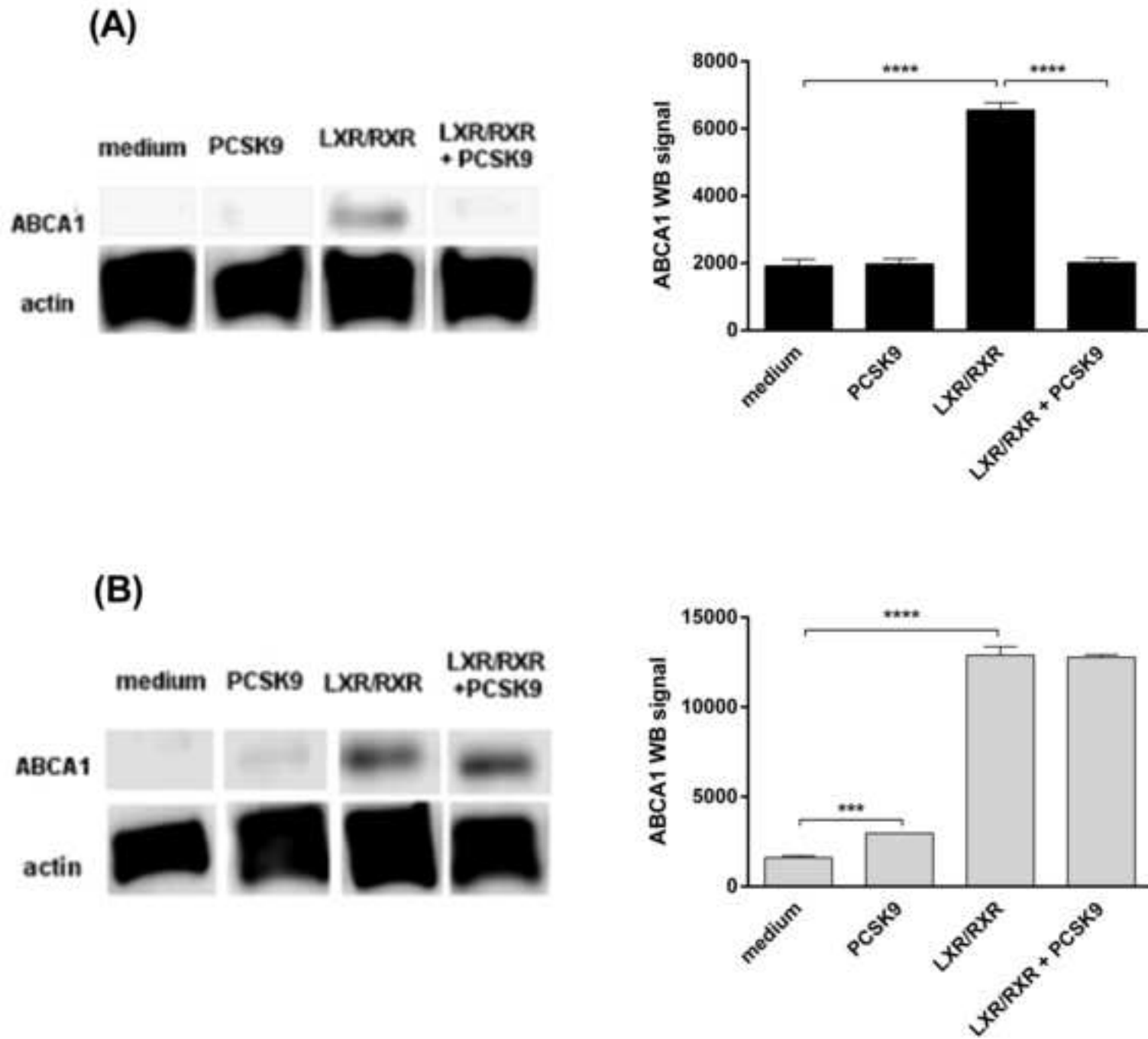


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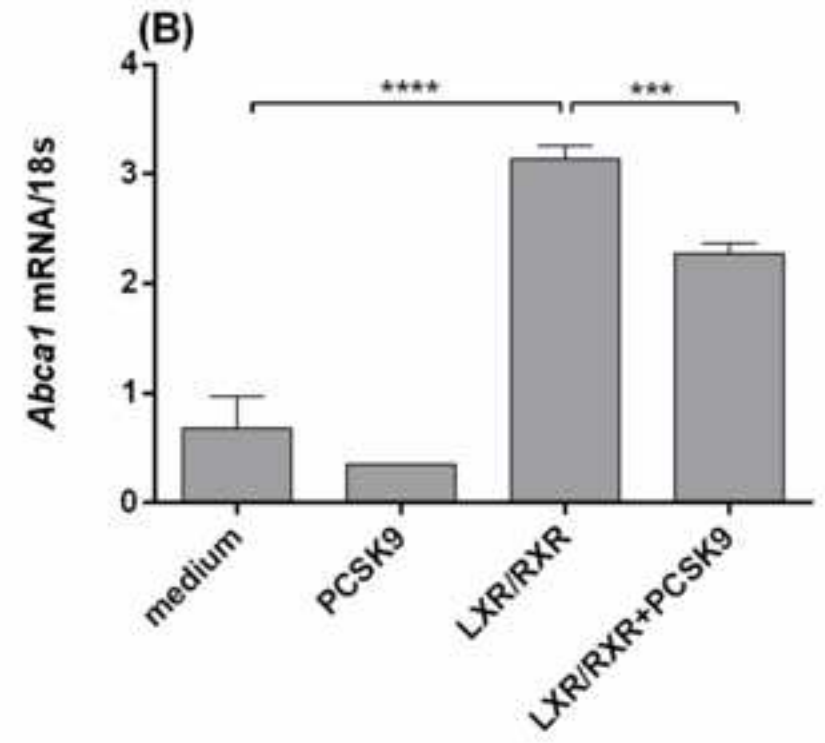
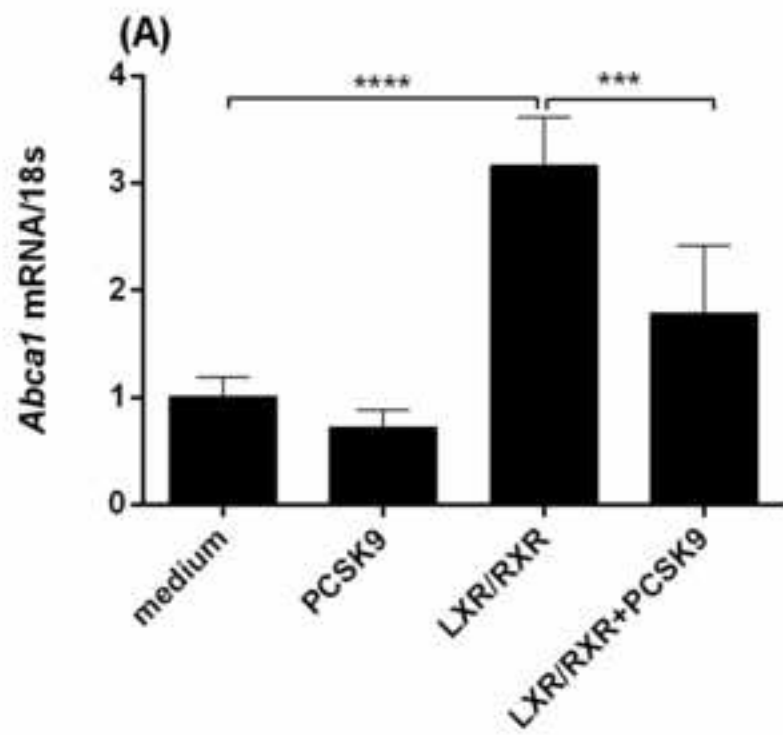


Figure 5
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