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Effect of anti-PCSK9 drugs on the association of PCSK9 to LDL

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein known to interact with the LDL receptor, thereby promoting its degradation and blunting the uptake of LDL from the circulation. In this context, anti-PCSK9 monoclonal antibodies (mAbs) and siRNAs have been approved for the treatment of hypercholesterolaemia. Previous studies have shown that a significant proportion of circulating PCSK9 is associated with LDL. The aim of our research is to investigate the effect of mAbs and siRNA on the association of PCSK9 protein with LDL. In this study, 10 statin-intolerant patients received treatment with anti-PCSK9 mAbs or siRNA, in addition to therapy with a low-dose statin and ezetimibe. Their plasma samples were analysed before and after 1, 3, and 6/9 months of treatment. The results showed that both the monoclonal antibodies and inclisiran reduced LDL-C levels by 50% to 60%. LDL-C levels decreased from 92±28 mg/dL to 44±26 mg/dL after siRNA treatment and reached 97±9, 27±10, 32±14, and 23±10 mg/dL after mAbs therapy. The circulating PCSK9 level decreased by 70% after the first siRNA injection, while it increased 10-fold after mAbs therapy. Regardless of treatment, the percentage of PCSK9 bound to LDL did not vary from baseline and remained constant during the treatment period. Whether this is of physiological relevance remains to be addressed.

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Background

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein that plays a crucial role in the regulation of plasma low-density lipoprotein cholesterol (LDL-C) levels [1]. It is primarily produced in the liver [2]. The PCSK9 protein binds to LDL receptors (LDLR) on the cell surface, leading to internalisation of the PCSK9-LDLR complex and targeting LDLR for lysosomal degradation, thus reducing the cell's ability to remove LDL-C from the bloodstream [3]. This leads to higher levels of LDL-C in the circulation and contributes to the development of atherosclerosis and cardiovascular disease [4, 5]. Lowering LDL-C levels is a key focus in the prevention and treatment of cardiovascular disease [6]. Lifestyle changes, including a healthy diet, regular exercise and avoiding tobacco use, can help to lower LDL-C levels. In addition, therapies such as statins are commonly prescribed to reduce LDL-C and lower the risk of cardiovascular events (7). The central role of PCSK9 in modulating LDL-C levels has driven the development of several approaches to inhibit this protein [8].

Evolocumab and alirocumab are fully humanised monoclonal antibodies (mAbs) that target circulating PCSK9 and have been investigated in several clinical trials, including the two outcome trials FOURIER and ODYSSEY OUTCOMES [9, 10]. Inclisiran is a small interfering RNA (siRNA) that specifically inhibits the hepatic synthesis of PCSK9 [11]. Both treatments lead to increased expression of LDLR in the liver, enhancing the removal of LDL-C from the blood [12, 13]. PCSK9 inhibitors are used as a therapeutic option to lower LDL-C levels in individuals with hypercholesterolaemia and a high risk of cardiovascular events [14]. Several studies are investigating the possible effects of PCSK9 inhibition beyond LDL-C levels [15]. In this study, we investigated the effect of mAbs and siRNA on the association of PCSK9 protein with LDL.

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Methods

Subjects and samples

For this study, we have selected 10 patients who were treated with a statin plus ezetimibe and experienced statin intolerance. Of these, 9 were classified as medium risk and 1 was classified as high risk for intolerance. Subsequently, the patients were divided into three groups, of which 5 subjects received a monoclonal antibody anti-PCSK9, 4 others received siRNA treatment, in addition to therapy with a low-dose statin and ezetimibe, and 1 patient replaced the statin with siRNA. Plasma samples were collected from each patient at baseline and after 1, 3 and 6/9 months of therapy. All blood samples were subjected to low-speed centrifugation (3000 rpm, 12 min) to obtain plasma to which the protease inhibitor (Halt™ Protease Inhibitor Cocktail, Thermo Fisher, Italy) was added. Each participant gave written informed consent for the study. This study was conducted in accordance with the Declaration of Helsinki.

Iodixanol density gradient ultracentrifugation

Lipoproteins were isolated from plasma using three layer-density of OptiPrep™ solvent as previously described [16].

Statistics

Statistical analyses were performed with GraphPad Prism 9.0. Data were analysed using the unpaired t-test and the one-way ANOVA test.

Results

The study cohort consisted of n=10 subjects, with 9 subjects being treated with alirocumab, evolocumab or inclisiran in addition to their existing therapy, while the remaining 1 subject was treated only with inclisiran. Plasma samples were collected before the therapies and at 1, 3, and 6/9 months (T1, T2 and T3, respectively) after the first injection. All samples were analysed for PCSK9 and lipoprotein distribution. Baseline levels of total cholesterol, LDL-C, HDL-C and TG of the subjects are reported in **Table 1**.

In patients receiving monoclonal antibody therapy, approximately 12% of the total PCSK9 was bound to LDL at baseline (T0). After one month of treatment with an anti-PCSK9 monoclonal antibody (T1), a 70% reduction in LDL-C levels (from 97±9 mg/dL to 27±10 mg/dL) was observed. Conversely, plasma PCSK9 levels increased 10-fold, from 562±156 ng/mL to 4,925±1400 ng/mL. Interestingly, the percentage of circulating PCSK9 bound to LDL remained unchanged throughout the therapy duration, despite the marked changes in LDL-C and PCSK9 levels. The data observed at T1 was confirmed at T2 and T3, where LDL-C levels were 27±10, 32±14 and 23±10 mg/dL, respectively; while PCSK9 concentration reached 4,925±1400, 5,360±755 and 5,843±920 ng/mL, respectively (**Figure 1**). The percentage of PCSK9 bound to LDL remained consistent with the level observed at T1, reaching values of 15% at both T2 and T3 (**Figure 1**).

Compared to anti-PCSK9 mAb therapy, patients treated with inclisiran showed a decrease in both LDL-C and PCSK9 plasma levels (from 92±28 mg/dL to 44±26 mg/dL and from 691±187 ng/mL to 212±63 ng/mL, respectively) one month after the first inclisiran injection (T1) (n=5; **Figure 2**). The calculated percentage of association between PCSK9 and LDL at T1 was comparable to that at T0. After three and nine months of inclisiran treatment (T2 and T3, respectively), the percentage of PCSK9 bound to LDL remained unchanged, with no further variations in LDL-C and PCSK9 levels.

Discussion

Several studies have shown that PCSK9 is associated with LDL in plasma [5, 17, 18]. To date, the nature and the physiologic role of the PCSK9 associated with LDL and other lipoproteins, such as Lp(a) [18], remains unclear. Some observations suggest that LDL-bound PCSK9 is the more functional form of this protein, as the interaction with an LDL particle protects PCSK9 from cleavage by furin and the protein remains bound to the particle in its active form [19]. On the other hand, in vitro studies showed that the addition of recombinant PCSK9 to LDL reduces the affinity of PCSK9 for LDLR, suggesting that LDL-bound PCSK9 is a less functional form of plasma PCSK9 [5, 20].

PCSK9 inhibitors (monoclonal antibodies and siRNA) have been

Table 1 | Report of total cholesterol (TC), HDL-C, LDL-C and TG in plasma patients before treatments.

Subjects	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	TG (mg/dL)
Patients with mAbs (N=5)	165±15	40±7	97±9	141±48
Patients with siRNA (N=5)	163±35	46±11	92±28	124±44

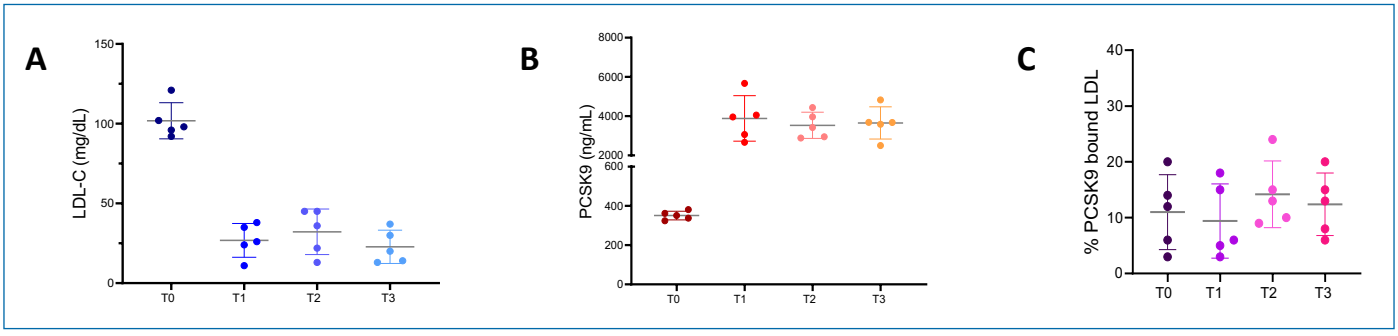

Figure 1 | Comparison among (A) LDL-C and (B) PCSK9 levels before (T0) and after (T1, T2, T3) anti-PCSK9 mAbs administration (n=5 for each group; p<0.005). (C) The percentage of circulating PCSK9 bound to LDL during the therapy (values are means±standard errors).

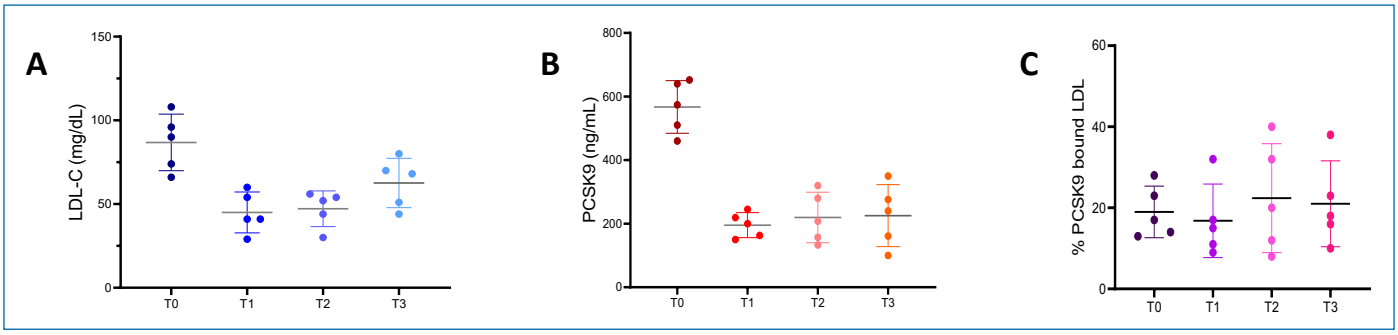

Figure 2 | Comparison among (A) LDL-C and (B) PCSK9 levels before (T0) and after (T1, T2, T3) inclisiran administration (n=5 for each group; p<0.005). (C) The percentage of circulating PCSK9 bound to LDL during the therapy (values are means±standard errors).

developed and approved for the treatment of hypercholesterolemia and are used in patients who need substantial reductions in their LDL-C levels to lower cardiovascular risk. In the present study, we aimed to investigate the association between PCSK9 and LDL particles in patients treated with two different anti-PCSK9 approaches.

According to the results from clinical trials [9, 10], we have demonstrated that both monoclonal antibodies and inclisiran reduce LDL-C levels by 50 to 60%. On the other hand, we clearly show that plasma PCSK9 levels differ significantly between the two therapies. In fact, mAbs increase plasma PCSK9 levels by up to 10-fold, which is due to the large amount of PCSK9 bound to the antibodies, whereas inclisiran reduces plasma PCSK9 protein levels by about 70%.

We found that while LDL-C levels were significantly reduced with both treatments, circulating PCSK9 levels behaved as expected (increased with the mAbs and decreased with inclisiran), while the percentage of PCSK9 bound to LDL did not vary from baseline and remained constant during the treatment period. Nevertheless, a relatively large amount of PCSK9 remains bound to LDL, especially after treatment with monoclonal antibodies. Whether this is of physiological relevance remains to be addressed. The nature of this association is currently being analysed to determine its status and whether the bound PCSK9 is structurally different from the unbound form. Preliminary observations from our ongoing study suggest that it is not the monoclonal antibody-bound form of PCSK9 that binds to lipoproteins, but the free form. Furthermore, we found that the lipoprotein-bound PCSK9 is not in the cleaved form (unpublished data). The mature form of PCSK9 (62 kDa) is believed to be more effective than the furin-cleaved form (55 kDa) in degrading the LDLR [21]; through western blotting analysis, we observed that the mature specifically associates with the LDL subfraction. This observation supports a previous finding that the PCSK9 species associated with LDL is primarily the intact heterodimer form, whereas the free PCSK9 (non-LDL-bound) is primarily in the furin-cleaved conformation [22]. It is tempting to speculate that the PCSK9-LDL-bound form can explain the biological function of LDLR, but further investigations are needed. It is worth noting that all subjects had previously been treated with statin therapy; therefore, the concentration of PCSK9 protein was elevated. In addition, the distribution of PCSK9 and its binding to lipoproteins may be influenced by prior therapy. Further studies on subjects before therapy are underway.

Our previous studies have investigated the interaction between PCSK9 and LDL [16], showing that high salt concentrations disrupt this binding, suggesting a non-covalent interaction. Moreover, our studies have explored the type of lipoproteins bound to PCSK9, uncovering a preference for a specific LDL subfraction. This particular subfraction, resembling remnant lipoproteins, exhibits increased buoyancy compared to mature LDL, characterised by enriched levels of apoE and apoCs, alongside elevated triglyceride content (unpublished data). This observation led us to hypothesise that PCSK9 may enter the bloodstream in association with VLDL, which are then metabolized to IDL, which could explain our finding. This possibility is currently being investigated in a specifically designed study. Overall, the association of lipoproteins with PCSK9 is thought to influence the PCSK9 activity towards the LDL receptor [23], which calls for further investigation into the potential biological significance of this LDL subfraction.

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Authorship and author contribution statement

SM wrote the MS and critically revised the data; VP and FME performed the laboratory work and collected all data; AP critically revised the data and wrote the MS; LG critically revised the data and read the MS; ALC designed the study, critically revised the data and wrote the MS.

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Conflict of interest

AL Catapano reports consulting fees/lecturing fees from Akcea, Amgen, Amryt, Sanofi, Esperion, Kowa, Novartis, Ionis Pharmaceuticals, Mylan, Menarini, Merck, Recordati, Regeneron Daiichi Sankyo, Genzyme, Aegerion, and Sandoz. The remaining authors have nothing to disclose.

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