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## **Treatment with PCSK9 inhibitors induces a more anti-atherogenic HDL lipid profile in patients at high cardiovascular risk**

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## Abstract

**Background:** Proprotein Convertase Subtilisin/Kexin type 9 inhibitors (PCSK9-I) have been reported to cause a moderate increase in high-density lipoprotein (HDL) cholesterol in human studies. We thus evaluated the effect of two approved PCSK9-I on the concentration and lipid composition of HDL particle subclasses.

**Subjects and methods:** 95 patients ( $62.8 \pm 10.3$  years old, 58% men), with or without statin and/or ezetimibe treatment and eligible for PCSK9-I therapy, received either evolocumab (140 mg) or alirocumab (75 or 150 mg). Their HDL particle profiles were measured by NMR spectroscopy at baseline and after 4 weeks of PCSK9-I treatment.

**Results:** PCSK9-I treatment increased the level of HDL-C by 7%. The level of medium-sized HDL particles (M-HDL-P) increased (+8%) while the level of XL-HDL-P decreased (-19%). The lipid core composition was altered in the smaller S- and M-HDL-P, with a reduction in triglycerides (TG) and an enrichment in cholesterol esters (CE), whereas the for the larger XL- and L-HDL-P the relative CE content decreased and the TG content increased. Ezetimibe therapy differentially impacted the HDL particle distribution, independently of statin use, with an increase in S-HDL-P in patients not receiving ezetimibe.

**Conclusions:** As S- and M-HDL-P levels are inversely related to cardiovascular risk, PCSK9-I treatment may result in a more atheroprotective HDL particle profile, particularly in patients not concomitantly treated with ezetimibe.

**Key Words:** High-density lipoprotein, dyslipidemia, drug, PCSK9, PSCK9 inhibition

**Abbreviations:** ApoA-I, apoprotein A-I; ApoB, apoprotein B; BMI, body mass index; CE, cholesterol esters; CETP, cholesteryl ester transfer protein; CVD, cardiovascular disease; CV, cardiovascular; FC, free cholesterol; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; HDL-CEC, HDL-dependent cholesterol efflux capacity; HDL-P, HDL particle; HeFH, heterozygous familial hypercholesterolemia; M-HDL-P, Medium-sized HDL particles; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDL-R, LDL receptor; L-HDL-P, Large-sized HDL particles; PCSK9, Proprotein Convertase Subtilisin/Kexin type 9; PCSK9-I, Proprotein Convertase Subtilisin/Kexin type 9 inhibitor; PL, phospholipids; S-HDL-P, Small-sized HDL particles; TG, triglycerides; VLDL, very-low-density lipoprotein; XL-HDL-P, Extra-Large-sized HDL particles.

## 1. Introduction

Proprotein Convertase Subtilisin/Kexin type 9 inhibitors (PCSK9-I) are the latest class of available therapies to reduce low-density lipoprotein cholesterol (LDL-C), and they have been shown to be effective at reducing the risk of cardiovascular disease (CVD) [1,2]. PCSK9 is a circulating protease that binds to the LDL receptor (LDL-R) and promotes its lysosomal degradation of the LDL receptor (LDL-R) in hepatocytes. PCSK9-I act by blocking the circulating PCSK9 protein. The consequent decrease in circulating PCSK9 activity results in an increase in LDL-R levels, thereby promoting the uptake of LDL by the liver and thus leading to a decrease in the level of circulating LDL cholesterol (LDL-C). Currently, the only approved PCSK9-I are two fully human monoclonal antibodies, alirocumab and evolocumab. In clinical trials, alirocumab and evolocumab, either alone or in combination with statins and/or other lipid-lowering therapies, have been shown to significantly reduce LDL-C levels (60% on average), depending on dosage [3]. These two PCSK9-I are currently recommended by the ESC/EAS to treat individuals with a very high CVD risk and who have been unable to sufficiently lower their LDL levels [4]. While PCSK9-I have been shown to be effective at reducing LDL-C levels, the limited number of studies to date have shown variable effects of PCSK9-I therapy on other lipoprotein classes, including high-density lipoprotein (HDL) [5]. HDL cholesterol (HDL-C) has been repeatedly inversely related to cardiovascular risk in most [6,7] but not all epidemiological studies [8]. Additional parameters beyond the cholesterol content of HDL particles (i.e., HDL-C) may help improve risk prediction. Indeed, HDL particles are heterogeneous in size and biochemical composition, and different HDL subpopulations may have different functional properties [9]. Nuclear magnetic resonance (NMR) spectroscopy was recently proposed as a reference tool to measure the concentration of total HDL particles (referred to as HDL-P), their size distribution, and lipid composition. To date, measurement of HDL particle profiles by NMR spectroscopy has been carried out in numerous cohort studies, and most have shown that small- and medium-sized HDL particles (referred to as S- and M-HDL-P) are inversely related to cardiovascular risk in various clinical settings [10–13]. In light of these results, NMR-based HDL-P measurement may improve risk prediction in cardiovascular high-risk populations receiving lipid-lowering pharmacotherapy, i.e. PCSK9-I.

To date, only four studies have analyzed the effects of PCSK9-I therapy on HDL particle profiles measured by NMR, with one study reporting no change [14], while the others found a moderate increase

in the total HDL-P level of approximately 10% [15–17]. However, the aforementioned studies were performed in patients from randomized controlled trials and were mostly restricted to analysis of the total HDL-P concentration. Hence, we have evaluated the effects of the two approved PCSK9-I, alirocumab and evolocumab, on the concentration and lipid composition of HDL subclasses, as measured by NMR spectroscopy, in high cardiovascular risk patients eligible for PCSK9-I therapy.

## **2. Patients and methods**

### ***2.1. Patients***

Ninety-five patients were consecutively selected from a prospective, open-label, single-center study conducted from 2016 until present at the Outpatient Lipid Clinic of the Charité Universitätsmedizin Berlin (Berlin, Germany). The selection period was from July 20<sup>th</sup>, 2016 until January 26<sup>th</sup>, 2017 and included all patients who participated in the study at this time, who provided written informed consent for an additional blood draw for the present analysis, and who had complete follow-up data of the second study visit (95 out of 133 patients). All patients received PCSK9-I (alirocumab at 75 or 150 mg sc once every 2<sup>nd</sup> week or evolocumab 140 mg sc once every 2<sup>nd</sup> week) in clinical routine. The inclusion criteria were age  $\geq$  18 years, prescription of PCSK9-I, and the ability to understand the purpose of the study. Patients receiving alirocumab or evolocumab according to directives from the Federal Joint Committee (“Gemeinsamer Bundesausschuss”) based on the 2016 European Society of Cardiology guidelines were eligible for inclusion in the study [18–20]. All patients received instructions from a physician regarding the use of PCSK9-I. PCSK9-I were prescribed in addition to statin therapy and other lipid-lowering therapies. The study involved two consultations, the first one took place before the start of the PCSK9-I treatment and the second one took place four weeks after treatment initiation (all of the patients received two PCSK9-I injections). At the first consultation, all patients completed a standard questionnaire regarding their medical history. In addition, they underwent a routine clinical examination. At both consultations, the patients underwent blood sampling following an overnight fast. Blood samples were collected in EDTA tubes for plasma isolation by centrifugation. Plasma aliquots were stored frozen at -80 °C until further use. Diabetes mellitus, hypertension, and CVD (coronary artery disease and/or cerebral artery disease and/or peripheral artery disease) were diagnosed based on the medical records. Written informed

consent was obtained from each patient included in the study; the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki and it was approved by the institution's ethics committees on research on humans (EA4/178/15 and 162/15). Statin intolerance was defined as the inability to tolerate  $\geq 3$  statins based on adverse events in the patients and/or objective parameters (i.e., increased levels of creatine kinase, aspartate aminotransferase, or alanine aminotransferase) [21,22].

## **2.2. Laboratory analyses**

All laboratory analyses were performed on fasting EDTA plasma samples. Concentrations of total cholesterol (TC), triglycerides (TG), LDL cholesterol (LDL-C) [beta-quantification], HDL cholesterol (HDL-C), and apolipoproteins A-I and B were measured on the same day of blood draw at a local laboratory (Labor Berlin, Berlin, Germany) as described previously [23]. The concentration of non-HDL cholesterol (non-HDL-C) was calculated by subtracting HDL-C from TC.

## **2.3. NMR-based HDL profiling**

NMR analyses were carried out on additional plasma EDTA aliquots that had been stored at  $-80\text{ }^{\circ}\text{C}$  until the biological analyses were carried. The number and lipid composition (cholesterol ester, triglycerides, phospholipids and free cholesterol) of HDL subclasses and mean HDL size were measured with a Nightingale high-throughput NMR metabolomics platform (Nightingale Ltd., Helsinki, Finland). Details of the experimental protocols have been described and reviewed elsewhere [24–27].

## **2.4. Statistical analyses**

Statistical analyses of data were performed using the SAS statistical software package (SAS Enterprise Guide Version 7.15; SAS Institute, Cary, NC, USA). Unless otherwise stated, data are presented as percentages for categorical (qualitative) variables and as means with standard deviation (SD) or 95% confidence intervals (CI) for quantitative data. The Shapiro-Wilks test was used to test the normality of the distribution of the residuals. The Chi-Square test was used to compare categorical variables. Individual changes ( $\Delta$ ) in lipid parameters were calculated as the difference between post- minus pre-treatment values and were analyzed using Student's paired *t*-tests when data were normally distributed. Comparisons between statin-taking versus statin-intolerant patients were made using Student's unpaired *t*-tests. Data were adjusted for concomitant lipid-lowering therapy by analysis of variance (ANOVA) followed by Tukey's *post-hoc* tests.

### 3. Results

#### 3.1. Patients characteristics at inclusion

The present cohort consisted of 95 patients receiving PCSK9-I (alirocumab at 75 or 150 mg sc once every 2<sup>nd</sup> week or evolocumab 140 mg sc once every 2<sup>nd</sup> week) in clinical routine. Patient characteristics and lipid values at baseline (i.e., at the initiation of PCSK9-I treatment) are presented in **Table 1** for the total cohort and according to the use of statins or ezetimibe. The total cohort consisted of 98.9% Caucasians, 57.9% men, and mean age was  $62.8 \pm 10.3$  years. Former CVD was recorded in 84.2% of patients, and 92.6% of patients had a very high risk of CVD according to the 2016 ESC guidelines [20]. Diabetes was diagnosed in 23.2% of patients, hypertension in 72.6% and 21.1% of patients were current smokers. In the total cohort, 51.6% of the patients were prescribed evolocumab and 48.4% alicumab. Almost half of patients (47.4%) received a statin treatment at baseline, while the other half (50.3%) was statin-intolerant and did not receive statins. Ezetimibe was used by half of the patients (50.5%) either in combination with statin therapy (80%) or as stand-alone lipid-lowering therapy (20%). Patients without statin therapy were older and less treated with ezetimibe than statin-taking patients. Similarly, patients without ezetimibe tended to be older and treated less with statins. Patients without concomitant ezetimibe therapy were more frequently treated with high-dose alicumab.

Regarding fasting lipid levels at baseline, TC, LDL-C, non-HDL-C and apoB levels were consistently lower in statin-taking and ezetimibe-taking patients as compared to the patients without statin or ezetimibe therapy (all  $p < 0.05$ , **Table 1**).

Regarding baseline values of HDL-related markers, HDL-C level was  $1.42 \pm 0.44$  mmol/L, while apoA-I, the major protein in HDL, was  $1.57 \pm 0.30$  g/L in the total cohort, without differences between statin-taking versus statin-intolerant or ezetimibe-taking versus ezetimibe-non-taking patients, respectively (**Table 1**).

The HDL particle profiles were determined by NMR-spectroscopy. At baseline, the average size of total HDL particles (mean HDL size) was  $9.57 \pm 0.18$  nm (**Table 2**). The mean concentration of total HDL-P was  $15737 \pm 2641$  nmol/L (**Table 2**). S-HDL-P represented the main subclass of HDL particles ( $10467 \pm 1421$  nmol/L), followed by M-HDL-P ( $3704 \pm 944$   $\mu$ mol/L) and then L-HDL-P ( $1340 \pm 720$  nmol/L) and XL-HDL-P ( $226 \pm 97$  nmol/L). The concentration of XL-HDL-P was significantly lower in the

statin-taking patients compared to statin-intolerant patients (–51 nmol/L [95% CI: –89; –12],  $p=0.01$ ), while there were no differences in baseline concentrations of other HDL particle subclasses, regardless of statin or ezetimibe usage (**Table 2**).

### **3.2. Effect of PCSK9-I on plasma lipids and apolipoproteins**

As shown in **Figure 1**, PCSK9-I treatment for 4 weeks led to a pronounced decrease in TC (–37.2%, –2.2 mmol/L [95% CI, –2.4; –2.1],  $p < 0.0001$ ) as well as in other pro-atherogenic lipids and apolipoproteins, including LDL-C (–53.5%, –2.2 mmol/L, [95% CI, –2.4; –2.0],  $p < 0.0001$ ), non-HDL-C (–50.8%, –2.2 mmol/L [95% CI: –2.4; –2.1],  $p < 0.0001$ ), fasting TG (–23.6%, –0.4 mmol/L [95% CI, –0.6; –0.3],  $p < 0.0001$ ), and apoB (–46.0%, –0.6 g/L [95% CI, –0.7; –0.6],  $p < 0.0001$ ). In terms of HDL-related parameters, HDL-C concentration increased by 7.0 % (+0.1 mmol/L [95% CI, 0.09; 0.11],  $p < 0.0001$ ) and apoA-I by 3.7% (+0.1 g/L [95% CI, 0.001; 0.1],  $p=0.0003$ ). None of the subjects reported any serious adverse events during follow-up treatment.

### **3.3. Effect of PCSK9-I on HDL-P subclasses and size**

Following PCSK9-I treatment for 4 weeks, mean HDL size remained unchanged as compared to baseline ( $p=0.49$ , **Figure 2A**), while total HDL-P tended to slightly increase (+2.4%, or +379 nmol/L [95% CI, –10; +768],  $p=0.06$ , **Figure 2B**). With regard to HDL particle subclasses, M-HDL-P increased by 8% (+326 nmol/L [95% CI, +202; +450],  $p < 0.0001$ , **Figure 2E**) and XL-HDL-P decreased by 19% (–43 nmol/L [95% CI, –58; –28],  $p < 0.0001$ , **Figure 2C**), while L-HDL-P and S-HDL-P levels remained unchanged (both  $p > 0.5$ , **Figures 2D and 2F**). Similar results were obtained when males and females were compared (supplementary Table 1). This was also the case for comparisons between alirocumab and evolocumab, except for changes in XL-HDL-P: here, evolocumab led to a significantly greater decrease compared to alirocumab (–56.8 versus –22.7 nmol/L,  $p=0.03$ ) (Supplementary Table 2). However, this effect was no longer significant when only evolocumab at 140 mg and alirocumab at 150 mg were compared ( $p=0.09$ ) without considering low-dose (75 mg) alirocumab.

### **3.4. Effect of PCSK9-I on lipid composition of HDL particles**

**Table 3** presents the relative lipid molecular composition, expressed as the percentage of total lipid, in each of the HDL particle subclasses at baseline and following PCSK9-I treatment (week 4). At baseline, lipid core composition (i.e., esterified cholesterol and triglycerides) differed for the various HDL



subclasses. For instance, M- and S-HDL-P had a higher CE content (36.2% and 37.9%, respectively) than XL- and L-HDL-P (32.7% and 32.4%, respectively). Conversely, M- and S-HDL-P had a lower TG content (5.7% and 5.9%, respectively) than XL and L-HDL-P (9.6% and 6.2%, respectively).

When changes in lipid composition between baseline and week 4 were compared, PCSK9-I treatment led to a relative decrease in CE in larger XL- and L-HDL-P of 1.4% and 2.1%, respectively (both  $p < 0.0001$ , **Table 3**), while CE increased in smaller M- and S-HDL-P by 1.2% and 1.1%, respectively, (both  $p < 0.0001$ ). Conversely, TG content increased in larger XL- and L-HDL-P by 3.1% and 0.9%, respectively, (both  $p < 0.0001$ ), while it decreased in smaller M- and S-HDL-P by 0.4% and 0.3%, respectively, (both  $p \leq 0.03$ ).

Upon PCSK9-I treatment, phospholipid (PL) and free cholesterol (FC) contents, which are the main HDL surface components, decreased in all of the HDL subclasses, except for XL-HDL-P, in which the FC content was unchanged, and for L-HDL-P, in which PL content increased by 1.8% ( $p < 0.0001$ , **Table 3**). Similar results were obtained when males and females were compared (supplementary Table 3), as well as for alirocumab *versus* evolocumab (supplementary Table 4).

### ***3.5. Effect of PCSK9-I treatment on HDL-related parameters with regard to concomitant lipid lowering therapy***

In the present cohort, 47% of patients received a concomitant statin therapy during PCSK9-I treatment, while 53% were not treated with statins (**Table 1**). Similarly, 51% of the patients received a concomitant ezetimibe therapy, while 49% did not receive ezetimibe while being treated with PCSK9-I. 37.9% of patients received a combination of both statin and ezetimibe treatment.

The effects of PCSK9-I treatment on mean HDL size and HDL particle concentrations according to concomitant lipid-lowering therapy are reported in **Figure 3** (statin vs. no statin) and **Figure 4** (ezetimibe vs. no ezetimibe). As reported for the entire cohort (**Figure 2**), no change was observed in mean HDL size with respect to concomitant statin or ezetimibe therapy (all  $p > 0.37$ , **Figures 3A and 4A**).

Total HDL-P remained unchanged in statin-taking patients ( $p=0.78$ ), while they increased in statin-intolerant patients by 637 nmol/L [95% CI, +192; +1,082] ( $p=0.006$ ), respectively, with no inter-group difference ( $p=0.17$ ; **Figure 3B**). Similarly, total HDL-P remained unchanged in the ezetimibe-taking patients ( $p=0.68$ ), while they increased in patients without ezetimibe treatment by 868 nmol/L [95% CI,

+277; +1,459] ( $p=0.005$ ), with an intergroup difference of 968 nmol/L [95% CI, 212; 1,725] ( $p=0.01$ , **Figure 4B**). The latter effect remained significant after further adjustment for concomitant statin therapy ( $p=0.04$ ).

When analyzing the effect of PCSK9-I treatment on the HDL particle subclasses according to statin treatment, similar changes were observed as for the entire cohort (**Figure 2**), with a decrease in the XL-HDL-P level in both statin-taking ( $-40$  nmol/L, [95% CI,  $-68$ ;  $-12$ ],  $p=0.007$ ) and statin-intolerant patients ( $-46$  nmol/L, [95% CI,  $-60$ ;  $-21$ ],  $p < 0.0001$ ; no inter-group difference,  $p=0.72$ , **Figure 3C**) and an increase in M-HDL-P in statin-taking patients ( $+225$  nmol/L, [95% CI,  $+26$ ;  $+425$ ],  $p=0.03$ ) and statin-intolerant patients ( $+417$  nmol/L, [95% CI,  $+262$ ;  $+571$ ],  $p < 0.0001$ ) with no inter-group difference,  $p=0.13$ , **Figure 3E**). Similarly, the concentration of XL-HDL-P decreased in both ezetimibe-taking patients ( $-36$  nmol/L, [95% CI,  $-63$ ;  $-8$ ],  $p=0.01$ ) and in patients without concomitant ezetimibe therapy ( $-45$  nmol/L, [95% CI,  $-60$ ;  $-31$ ],  $p < 0.0001$ ; no inter-group difference,  $p=0.53$ , **Figure 4C**), while the concentration of M-HDL-P increased in both the ezetimibe-taking patients ( $+199$  nmol/L, [95% CI,  $+48$ ;  $+351$ ],  $p=0.01$ ) and the non-ezetimibe-taking patients ( $+456$  nmol/L, [95% CI,  $+259$ ;  $+652$ ],  $p < 0.0001$ ). However, the increase in the concentration of M-HDL-P was greater in the patients without concomitant ezetimibe therapy, as compared to ezetimibe-taking patients (inter-group difference: 256 nmol/L [95% CI, 12; 500],  $p=0.04$ , **Figure 4E**). However, in sensitivity analyses, the latter effect did not remain significant after further adjustment for concomitant statin therapy ( $p=0.15$ ).

As for the entire cohort (**Figure 2**), the concentrations of L- and S-HDL-P remained unchanged in patients who did and who did not take statins, with no inter-group differences (all  $p > 0.07$ , **Figures 3D and 3F**). Similarly, the concentration of L-HDL-P remained unchanged with regard to ezetimibe therapy, with no intergroup difference ( $p=0.27$ , **Figure 4D**). However, in patients without ezetimibe therapy, S-HDL-P increased by 3.7% ( $+384$  nmol/L [95% CI,  $+39$ ;  $+729$ ],  $p=0.03$ ), while it remained unchanged in the ezetimibe-taking patients ( $p=0.17$ ), with an inter-group difference of 609 nmol/L [95% CI, 140;  $+1,079$ ] ( $p=0.01$ , **Figure 4F**). In sensitivity analyses, the latter effect remained significant after further adjustment for concomitant statin therapy ( $p=0.04$ ).

#### 4. Discussion

The present study confirmed that patients at high cardiovascular risk treated for four weeks with PCSK9-I displayed a major decrease in the levels of total cholesterol and atherogenic lipids, including LDL-C and non-HDL-C, as well as apoB. A moderate but significant increase in HDL-C and apoA-I was also observed, which is an effect of PCSK9-I that has been previously reported in a number of clinical trials, albeit with some discrepancies [14,15,28–32]. Although the level of HDL-C has been repeatedly inversely related to cardiovascular risk in epidemiological studies, a single measurement of HDL-C does not necessarily reflect the functionality of HDL particles and their pleiotropic atheroprotective properties. Indeed, HDL particles are heterogeneous in size and biochemical composition, and HDL subpopulations may have different functional properties [9].

We found that PCSK9 inhibition in hypercholesterolemic patients led to an increase in the concentration of M-HDL-P that was conversely associated with a decrease in the concentration of XL-HDL-P, thus resulting in no net change in the total concentration of HDL particles. Several studies have previously analyzed association between HDL particle subclasses, measured by NMR, and cardiovascular risk. While a few have reported a negative association with L-HDL-P [33–35], most have found S- and M-HDL-P to be independently associated with a lower cardiovascular risk in various clinical settings and to have a better prognostic value than HDL-C in high cardiovascular risk patients [11–13,36–40]. Treatment with PCSK9-I may, therefore, result in a more atheroprotective HDL particle profile.

To date, only two studies have measured HDL particle concentrations by NMR spectroscopy in hypercholesterolemic patients treated the two currently approved PCSK9-I, namely 420 mg of evolocumab monthly for 52-weeks [32] and 150 mg of alirocumab bi-weekly for a period of 12 weeks [15]. They reported an increase in the concentration of total HDL-P of 9.4% and 11.2%, respectively, while in the present study HDL-P concentration increased by 2.4% ( $p=0.06$ ) upon PCSK9-I treatment for four weeks. Thus, a longer treatment period and/or higher dose (or exposure) might be more effective at increasing the total HDL-P concentration.

This is the first study to analyze the effects of PCSK9-I on the lipid composition of HDL particle subclasses by NMR spectroscopy. Upon PCSK9-I treatment, the lipid core composition was altered in the smallest S- and M-HDL-P, with a reduction in TG and an enrichment in CE, whereas, conversely, the

larger XL- and L-HDL-P exhibited a decrease in their relative CE content and an increase in the TG content. To explain these changes in the lipid core composition of HDL subclasses, we hypothesize that the lower availability of apoB-containing particles in serum, as a consequence of PCSK9 inhibition, is likely to impair the action of the cholesteryl ester transfer protein (CETP) in reciprocal exchanges of HDL-cholesterylesters for triacylglycerols from apoB-containing lipoproteins [41]. Thus, the effects on the HDL profile observed under PCSK9 inhibition are probably an indirect consequence of the pronounced decrease in apoB-containing lipoproteins. However, Girona *et al.* observed a positive and independent association between the circulating PCSK9 concentration and plasma CETP activity in patients at high risk of a cardiovascular event, suggesting that PCSK9 and CETP could also share some molecular regulatory mechanisms that still need to be elucidated [42]. Interestingly, a recent Mendelian randomization study, based on a CETP genetic score, suggests that CETP activity is associated with a higher TG content specifically in S- and M-HDL-P [43]. This latter observation supports the hypothesis that PCSK9-I treatment induces a metabolism-dependent decrease in plasma CETP activity that leads to a relatively lower TG and higher CE content in S- and M-HDL-P. This could contribute to the maintenance or increase of these small HDL particles upon PCSK9-I therapy as they are less degraded by hepatic or endothelial lipases, because of their lower triacylglycerol content [44]. In addition, the CE enrichment in the core of S- and M-HDL-P may reflect an increased HDL-dependent cholesterol efflux capacity (HDL-CEC), since these particles behave as high-affinity acceptors of ATP Binding Cassette Subfamily A Member 1 (ABCA1)-mediated free cholesterol efflux from macrophages to HDL, thus leading to subsequent cholesterol esterification by lecithin-cholesterol acyltransferase (LCAT) [45,46].

Concomitant statin therapy did not influence the effect of PCSK9-I on the distribution of HDL particle subclasses. On the other hand, our study revealed that ezetimibe therapy could differentially impact HDL particle distribution. Indeed, patients who were not treated with ezetimibe displayed a more favorable HDL subclass profile following PCSK9-I treatment, notably with an increase in S-HDL-P, while this profile was unchanged in patients treated with ezetimibe. This increase was independent of statin therapy status and translated into an increase in total HDL-P level. Ezetimibe monotherapy treatment has been reported to specifically reduce the concentration of small HDL particles, without affecting the concentration of other HDL subclasses [47]. Given that dietary cholesterol significantly contributes to

nascent HDL that are produced by the intestine [48], this effect could be attributed to the ezetimibe-induced inhibition of intestinal cholesterol absorption. Since S-HDL-P have been reported to represent the most potent anti-atherogenic HDL subclass [14,15,28–32,49], further follow-up studies of patients treated with PCSK9-I are required to investigate the impact of concomitant ezetimibe treatment on long-term prognosis.

The present study is the first to assess the effect of PCSK9-I on HDL particles by in-depth HDL profiling, including the concentration of total HDL particles as well as the proportion of 4 HDL subclasses (XL-, L-, M-, and S-HDL-P) and their individual lipid compositions. However, there are limitations to our study. It included a low number of participants treated for one month with PCSK9-I, and thus, further long-term follow-up studies are required to confirm whether these effects are maintained or amplified over long-term treatments and to determine their specific impact on long-term CVD risk. It was an observational study and we did not assess the activity of HDL-associated enzymes contributing to HDL remodeling such as CETP and LCAT neither HDL functionality such as cholesterol efflux capacity. Thus, the translation of these HDL profile changes into HDL atheroprotective functions deserves further investigation.

## **5. Conclusions**

We here report that, in addition to its well-established hypocholesterolemic effect, PCSK9 inhibition using clinically approved monoclonal antibodies in hypercholesterolemic patients can also favorably affect the distribution of HDL subclasses, especially in patients who do not receive concomitantly ezetimibe. Although the mechanisms underlying these effects still need to be clarified, they are potentially caused by reduced levels of apoB-containing lipoproteins in serum as acceptors of cholesterol from HDL, which is an effect that could be partially concealed by ezetimibe-induced inhibition of intestinal cholesterol absorption. Further exploration is needed to determine whether the reported effects of PCSK9 inhibition on HDL particle profiles associate with reduced cardiovascular risk.

**Author contributions:** **Cécile Ingueneau** designed the study, analyzed the data, interpreted the results, wrote the manuscript and approved the final manuscript as submitted. **Tim Hollstein** designed the study, collected the data, interpreted the results, wrote the manuscript and approved the final manuscript as submitted. **Thomas Grenkowitz** collected the data, interpreted the results, revised the manuscript and approved the final manuscript as submitted. **Jean-Bernard Ruidavets** analyzed the data, interpreted the results, revised the manuscript and approved the final manuscript as submitted. **Ursula Kassner** designed the study, collected the data, revised the manuscript and approved the final manuscript as submitted. **Thibaut Duparc** analyzed the data and approved the final manuscript as submitted. **Guillaume Combes** collected the data and approved the final manuscript as submitted. **Bertrand Perret** interpreted the results, revised the manuscript and approved the final manuscript as submitted. **Annelise Genoux** interpreted the results, revised the manuscript and approved the final manuscript as submitted. **Friederike Schumann** collected the data, revised the manuscript and approved the final manuscript as submitted. **Thomas Bobbert** interpreted the results, revised the manuscript and approved the final manuscript as submitted. **Elisabeth Steinhagen-Thiessen** designed the study, interpreted the results revised the manuscript and approved the final manuscript as submitted. **Laurent O. Martinez** conceived, designed and supervised the study, wrote the manuscript and approved the final manuscript as submitted.

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## Tables

**Table 1 – Baseline characteristics of the study cohort**

|  | <b>TOTAL<br/>(n = 95)</b> | <b>Statin-taking patients<br/>(n = 45)</b> | <b>Patients without statins<br/>(n = 50)</b> | <b>Ezetimibe-taking patients<br/>(n = 48)</b> | <b>Patients without<br/>ezetimibe (n = 47)</b> |
|--|---------------------------|--|--|---|--|
| Male, n (%)                                      | 55 (57.9)                 | 29 (64.4)                                  | 26 (52.0)                                    | 29 (60.4)                                     | 26 (55.3)                                      |
| Caucasians, n (%)                                | 94 (98.9)                 | 44 (97.8)                                  | 50 (100.0)                                   | 47 (97.9)                                     | 47 (100.0)                                     |
| Age (years)                                      | 62.8 ± 10.3               | 59.4 ± 9.7                                 | 65.8 ± 10.0*                                 | 60.9 ± 10.2                                   | 64.7 ± 10.3                                    |
| BMI, kg/m <sup>2</sup>                           | 27.2 ± 5.0                | 27.5 ± 4.7                                 | 27.0 ± 5.3                                   | 27.4 ± 4.9                                    | 27.1 ± 5.2                                     |
| <b><u>Clinical characteristics</u></b>           |                           |  |  |   |  |
| CVD, n (%)                                       | 80 (84.2)                 | 40 (88.9)                                  | 40 (80.0)                                    | 42 (87.5)                                     | 38 (80.1)                                      |
| Very high CVD risk, n (%)                        | 88 (92.6)                 | 43 (95.6)                                  | 45 (90.0)                                    | 46 (95.8)                                     | 42 (89.4)                                      |
| Diabetes, n (%)                                  | 22 (23.2)                 | 10 (22.2)                                  | 12 (24.0)                                    | 12 (25.0)                                     | 10 (21.3)                                      |
| Hypertension, n (%)                              | 69 (72.6)                 | 35 (77.8)                                  | 34 (68.0)                                    | 37 (77.1)                                     | 32 (68.1)                                      |
| Current/former smoker, n (%)                     | 20 (21.1)                 | 9 (20.0)                                   | 11 (22.0)                                    | 9 (18.8)                                      | 11 (23.4)                                      |
| <b><u>Concomitant lipid-lowering therapy</u></b> |                           |  |  |   |  |
| Statin treatment, n (%)                          | 45 (47.4)                 | 45 (100.0)                                 | 0 (0.0)                                      | 36 (75.0)                                     | 9 (19.2)*                                      |
| Ezetimibe treatment, n (%)                       | 48 (50.5)                 | 36 (80.0)                                  | 12 (24.0)*                                   | 48 (100.0)                                    | 0 (0.0)  |
| <b><u>Prescribed PCSK9-I</u></b>                 |                           |  |  |   |  |
| Evolocumab 140 mg, n (%)                         | 49 (51.6)                 | 25 (55.6)                                  | 24 (48.0)                                    | 26 (54.2)                                     | 23 (48.9)                                      |
| Alirocumab 150 mg, n (%)                         | 23 (24.2)                 | 7 (15.6)                                   | 17 (34.0)                                    | 7 (14.6)                                      | 17 (36.2)*                                     |
| Alirocumab 75 mg, n (%)                          | 23 (24.2)                 | 13 (28.9)                                  | 9 (18.0)                                     | 15 (31.3)                                     | 7 (14.9)                                       |
| <b><u>Lipid parameters</u></b>                   |                           |  |  |   |  |
| Total cholesterol (mmol/L)                       | 6.02 ± 1.55               | 5.26 ± 1.31                                | 6.69 ± 1.43*                                 | 5.47 ± 1.59                                   | 6.59 ± 1.29*                                   |
| LDL-C (mmol/L)                                   | 4.11 ± 1.41               | 3.43 ± 1.20                                | 4.71 ± 1.32*                                 | 3.62 ± 1.44                                   | 4.62 ± 1.20*                                   |

|                        |             |             |              |             |              |
|------------------------|-------------|-------------|--------------|-------------|--------------|
| Non-HDL-C (mmol/L)     | 4.50 ± 1.54 | 3.81 ± 1.32 | 5.11 ± 1.46* | 3.97 ± 1.54 | 5.06 ± 1.33* |
| HDL-C (mmol/L)         | 1.42 ± 0.44 | 1.38 ± 0.40 | 1.45 ± 0.47  | 1.41 ± 0.44 | 1.43 ± 0.44  |
| Triglycerides (mmol/L) | 1.84 ± 1.29 | 1.71 ± 1.24 | 1.97 ± 1.33  | 1.70 ± 1.17 | 2.00 ± 1.40  |
| ApoB (g/L)             | 1.34 ± 0.38 | 1.20 ± 0.34 | 1.46 ± 0.38* | 1.24 ± 0.40 | 1.44 ± 0.34* |
| ApoA-I (g/L)           | 1.57 ± 0.30 | 1.51 ± 0.27 | 1.62 ± 0.33  | 1.54 ± 0.32 | 1.60 ± 0.29  |

For categorical variables, values are presented as number with frequency (%) in parentheses. For continuous variables, values are expressed as mean ± SD.

\*:  $p < 0.05$  between patients who did or who did not take statins (middle two columns) or between patients who did or who did not take ezetimibe (right two columns), tested by the unpaired Students  $t$ -test for continuous and normally distributed variables and by the  $\chi^2$  test for categorical variables.

**Table 2 – Baseline HDL size and subclass particles numbers**

|                      | <b>TOTAL<br/>(n = 95)</b> | <b>Statin-taking patients<br/>(n = 45)</b> | <b>Patients without statin<br/>(n = 50)</b> | <b>Ezetimibe-taking patients<br/>(n = 48)</b> | <b>Patients without ezetimibe<br/>(n = 47)</b> |
|----------------------|---------------------------|--|---|---|--|
| Mean HDL size (nm)   | 9.57 ± 0.18               | 9.55 ± 0.16                                | 9.59 ± 0.20                                 | 9.56 ± 0.17                                   | 9.58 ± 0.18                                    |
| Total HDL-P (nmol/L) | 15737 ± 2641              | 15495 ± 2741                               | 15955 ± 2557                                | 15983 ± 2617                                  | 15486 ± 2671                                   |
| XL-HDL-P (nmol/L)    | 226 ± 97                  | 199 ± 80                                   | 249 ± 105*                                  | 215 ± 103                                     | 243 ± 89                                       |
| L-HDL-P (nmol/L)     | 1340 ± 720                | 1253 ± 594                                 | 1419 ± 815                                  | 1322 ± 720                                    | 1383 ± 716                                     |
| M-HDL-P (nmol/L)     | 3704 ± 944                | 3621 ± 949                                 | 3779 ± 943                                  | 3784 ± 961                                    | 3622 ± 930                                     |
| S-HDL-P (nmol/L)     | 10467 ± 1421              | 10422 ± 1678                               | 10508 ± 1159                                | 10666 ± 1,437                                 | 10264 ± 1,391                                  |

Values are expressed as means ± SD.

\*: p < 0.05 between patients who did or who did not receive statins (middle two columns) or between patients who did or who did not take ezetimibe (right two columns), tested by the unpaired Students *t*-test for continuous and normally distributed variables.



**Table 3 – Changes in lipid composition of HDL particle subclasses after PCSK9-I therapy**

|                      |                    | Pre-treatment lipid composition (%) | Post-treatment lipid composition (%) | Change ( $\Delta$ ) after four weeks PCSK9-I treatment (%) | P-value           |
|----------------------|--------------------|-------------------------------------|--------------------------------------|--|-------------------|
| <b><u>XL-HDL</u></b> |                    |                                     |                                      |  |                   |
| Core                 | Cholesterol esters | 32.7 $\pm$ 3.4                      | 31.3 $\pm$ 3.8                       | <b>-1.4 (-2.1, -0.6)</b>                                   | <b>0.0006</b>     |
|                      | Triglycerides      | 9.6 $\pm$ 4.2                       | 12.7 $\pm$ 7.5                       | <b>+3.1 (+1.9, +4.3)</b>                                   | <b>&lt;0.0001</b> |
| Surface              | Phospholipids      | 44.9 $\pm$ 7.4                      | 43.2 $\pm$ 10.8                      | <b>-1.7 (-3.2, -0.2)</b>                                   | <b>0.03</b>       |
|                      | Free cholesterol   | 12.8 $\pm$ 2.8                      | 12.8 $\pm$ 3.8                       | -0.1 (-0.6, +0.5)  | 0.77              |
| <b><u>L-HDL</u></b>  |                    |                                     |                                      |  |                   |
| Core                 | Cholesterol esters | 32.4 $\pm$ 5.0                      | 30.3 $\pm$ 6.2                       | <b>-2.1 (-3.0, -1.1)</b>                                   | <b>&lt;0.0001</b> |
|                      | Triglycerides      | 6.2 $\pm$ 2.6                       | 7.1 $\pm$ 3.7                        | <b>+0.9 (+0.4, +1.5)</b>                                   | <b>0.0005</b>     |
| Surface              | Phospholipids      | 56.1 $\pm$ 4.4                      | 57.9 $\pm$ 4.2                       | <b>+1.8 (+1.1, +2.6)</b>                                   | <b>&lt;0.0001</b> |
|                      | Free cholesterol   | 5.4 $\pm$ 0.8                       | 4.6 $\pm$ 0.8                        | <b>-0.7 (-0.9, -0.6)</b>                                   | <b>&lt;0.0001</b> |
| <b><u>M-HDL</u></b>  |                    |                                     |                                      |  |                   |
| Core                 | Cholesterol esters | 36.2 $\pm$ 5.3                      | 37.4 $\pm$ 4.2                       | <b>+1.2 (+0.6, +1.8)</b>                                   | <b>0.0003</b>     |
|                      | Triglycerides      | 5.7 $\pm$ 2.6                       | 5.3 $\pm$ 2.0                        | <b>-0.4 (-0.7, -0.001)</b>                                 | <b>0.03</b>       |
| Surface              | Phospholipids      | 53.6 $\pm$ 3.1                      | 53.0 $\pm$ 2.6                       | <b>-0.6 (-1.0, -0.2)</b>                                   | <b>0.002</b>      |
|                      | Free cholesterol   | 4.5 $\pm$ 0.4                       | 4.3 $\pm$ 0.4                        | <b>-0.2 (-0.3, -0.1)</b>                                   | <b>&lt;0.0001</b> |
| <b><u>S-HDL</u></b>  |                    |                                     |                                      |  |                   |
| Core                 | Cholesterol esters | 37.9 $\pm$ 3.8                      | 39.0 $\pm$ 3.2                       | <b>+1.1 (+0.6, +1.6)</b>                                   | <b>&lt;0.0001</b> |
|                      | Triglycerides      | 5.9 $\pm$ 2.0                       | 5.5 $\pm$ 1.6                        | <b>-0.3 (-0.6, -0.001)</b>                                 | <b>0.02</b>       |
| Surface              | Phospholipids      | 51.1 $\pm$ 2.2                      | 50.6 $\pm$ 2.0                       | <b>-0.5 (-0.8, -0.1)</b>                                   | <b>0.007</b>      |
|                      | Free cholesterol   | 5.2 $\pm$ 0.4                       | 4.9 $\pm$ 0.4                        | <b>-0.3 (-0.4, -0.2)</b>                                   | <b>&lt;0.0001</b> |

Pre- and post-treatment lipid composition of HDL particles with phospholipid, cholesterol esters, free cholesterol, and triglyceride contents in XL-, L-, M-, and S-HDL particles expressed as arithmetic mean  $\pm$  standard deviation. Changes in lipid components ( $\Delta$ ) after four weeks of PCSK9-I treatment are expressed as arithmetic means with their 95% confidence interval. Statistical significance was determined by the paired Students *t*-test. Significant changes are highlighted in bold.

**Figure 1. Changes in lipid parameters in the full cohort after four weeks of PCSK9-I therapy.**

The absolute changes in plasma concentration of total cholesterol, LDL-C, non-HDL-C, HDL-C, triglycerides, apoB, and apoA-I after four weeks of PCSK9-I therapy were calculated as post- minus pre-treatment concentration. Statistical significance was determined by one-sample *t*-tests, and significant changes are displayed in bold. Percentage changes were calculated as the average post-treatment

concentration divided by the average pre-treatment concentration multiplied by 100 minus 100. Error bars denote the 95% confidence interval.

**Figure 2. Changes in mean HDL size and HDL particle concentrations in the full cohort after four weeks of PCSK9-I therapy.**

Absolute changes in (A) mean HDL size, (B) total HDL-P, (C) XL-HDL-P, (D) L-HDL-P, (E) M-HDL-P, and (F) S-HDL-P after four weeks of PCSK9-I therapy were calculated as post- minus pre-treatment values. Statistical significance was determined by paired *t*-tests. Percentage changes were calculated as the average post-treatment value divided by the average pre-treatment value multiplied by 100 minus 100. Error bars denote the 95% confidence interval.

**Figure 3. Changes in mean HDL size and HDL particle concentrations after four weeks of PCSK9-I therapy according to statin therapy status.**

Absolute changes in (A) mean HDL size, (B) total HDL-P, (C) XL-HDL-P, (D) L-HDL-P, (E) M-HDL-P, and (F) S-HDL-P after four weeks of PCSK9-I therapy were calculated as post- minus pre-treatment values and are shown in statin-taking (black bar) and statin-intolerant (white bar) patients. Statistical significance between both groups was determined by Student's unpaired *t*-tests. Error bars denote the 95% confidence interval.

**Figure 4. Changes in mean HDL size and HDL particle concentrations after four weeks of PCSK9-I therapy according to ezetimibe therapy status.**

Absolute changes in (A) mean HDL size, (B) total HDL-P, (C) XL-HDL-P, (D) L-HDL-P, (E) M-HDL-P, and (F) S-HDL-P after four weeks of PCSK9-I therapy were calculated as post- minus pre-treatment values and are shown for ezetimibe-taking (black bar) and ezetimibe-non-taking (white bar) patients. Statistical significance between both groups was determined by Student's unpaired *t*-test. Error bars denote the 95% confidence interval.

## **Highlights**

- PCSK9-I treatment results in a more atheroprotective HDL particle profile as:
- it decreases plasma levels of extra-large HDL particles.
- it increases plasma levels of medium-sized HDL particles.
- it increases small HDL particle levels in patients not receiving ezetimibe.

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**Submission declaration for Ingueneau et al.**

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Sincerely yours,

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**Declaration of interests for Ingueneau et al.**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

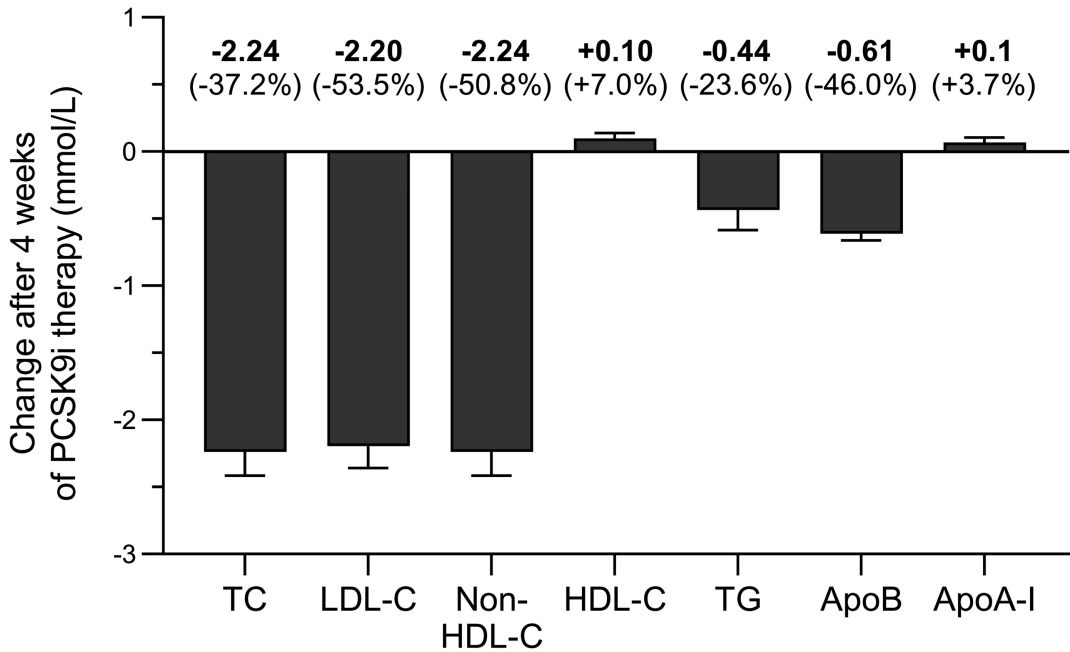


Figure 1