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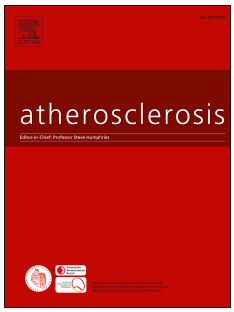

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Circulating PCSK9 levels are not associated with the severity of hepatic steatosis and NASH in a high-risk population

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ABSTRACT

Background and aims: Some studies suggested that proprotein convertase subtilisin kexin type 9 (PCSK9) is linked to liver steatosis severity and non-alcoholic steatohepatitis (NASH). We aimed to assess whether circulating PCSK9 levels are associated with either liver fat content (LFC) or histological markers of NASH in high-risk patients.

Methods: We present results from three cross-sectional studies from two French Hospitals: Dijon and Numevox (departments of Endocrinology) and Angers (department of Hepatology). Only patients without lipid-lowering therapy were included. All 132 patients had type 2 diabetes in Dijon, compared to 55/224 in Numevox (25%) and 39/122 in Angers (32%). LFC was assessed on MRI (Dijon and Numevox), and NASH lesion on liver biopsy (Angers). Additionally, we included mRNA results from 138 overweight patients in a Belgian Hospital (Antwerp).

Results: While circulating levels of PCSK9 were positively correlated with total cholesterol, LDL-C, triglycerides and non-HDL-C in all 3 cohorts, no significant association was found between PCSK9 and transaminases. Furthermore, no association was found between plasma PCSK9 levels and LFC in both Numevox ($\beta_{adjusted}$=0.71±1.33, p=0.60) and Dijon ($\beta_{adjusted}$=-1.03±0.90, p=0.25). There was no correlation between circulating PCSK9 and histological liver lesions: steatosis severity ($\beta_{adjusted}$=-3.95±2.75, p=0.15), NASH activity score ($\beta_{adjusted}$=-0.31±0.17, p=0.082), lobular (β=-0.067±0.055, p=0.22) or portal inflammation (β=-0.088±0.079, p=0.27), ballooning (β=-0.025±0.065, p=0.70) and fibrosis (β=-0.17±0.11, p=0.12). Finally, hepatic PCSK9 mRNA levels were not correlated with NASH histological severity.

Conclusions: Circulating PCSK9 concentrations are not associated with the severity of liver steatosis or histological markers of NASH. These data are reassuring regarding the clinical use of PCSK9 inhibitors in cardiovascular diseases.

1. Introduction

Proprotein convertase subtilisin kexin type 9 (PCSK9) has emerged as a critical regulator of cholesterol homeostasis, mainly by acting as an inhibitor of the LDL receptor (LDLR) pathway [1]. Once secreted by the hepatocyte, circulating PCSK9 is able to bind to the extra-cellular EGF(A) domain of the LDLR and to promote its lysosomal degradation, thereby reducing LDLR recycling at the cell surface and increasing plasma LDL-cholesterol (LDL-C) levels [2]. Beside this extracellular PCSK9 pathway, some data suggest that PCSK9 can directly interfere intracellularly with LDLR trafficking by an incompletely understood pathway [3].

The discovery of PCSK9 has quickly led to the development of PCSK9 inhibitors for the pharmacological management of hypercholesterolemia and cardiovascular diseases (CVD) [4]. PCSK9 human monoclonal antibodies (mAb), that efficiently block the extra-cellular PCSK9 pathway, significantly reduced major cardiovascular events in dedicated cardiovascular outcomes trials on top of statin therapy [5]. Importantly, there was no specific safety concern with PCSK9 mAb, even in patients who achieved very low (*i.e.* < 0.2 mmol/L) LDL-C levels [6].

Non-alcoholic fatty liver disease (NAFLD) is becoming the most important cause of liver disease worldwide. Epidemiology of NAFLD parallels to the prevalence of obesity and it is acknowledged as the liver complication of metabolic syndrome [7]. NAFLD is a spectrum of progressive liver diseases that ranges from simple steatosis, over steatohepatitis (NASH) to fibrosis that can lead to cirrhosis and hepatocellular carcinoma.

Yet, some interplay has been underscored between cholesterol metabolism and the pathogenesis of NASH [8, 9].

For instance, the occurrence of liver steatosis has been identified as a safety concern during the development of some hypocholesterolemic drugs, such as lomitapide and mipomersen. Lomitapide inhibits the microsomal triglyceride transfer protein (MTP) and therefore blocks the assembly and secretion of apolipoprotein B (apoB)-containing lipoproteins, leading to hepatic steatosis in phase II and III clinical trials [10]. Similar safety issue was observed

with mipomersen, a second-generation antisense oligonucleotide that inhibits hepatic apoB-100 synthesis and secretion [11]. In accordance with *in vivo* lipoprotein kinetic studies performed in healthy volunteers which demonstrate that the hypocholesterolemic effect of PCSK9 mAb is mostly related to an increased clearance of LDL-apoB, with a modest decrease of LDL-apoB production [12, 13], no liver safety signal was reported during the clinical development of PCSK9 mAb.

However, some observational studies suggest a potential link between PCSK9 and hepatic steatosis. For instance, we showed that plasma PCSK9 concentrations are associated with hepatic insulin resistance and liver steatosis in young healthy volunteers fed a high-fructose diet [14]. In addition, Ruscica *et al.* found that circulating PCSK9 levels were positively associated with histological markers of non-alcoholic steatohepatitis (NASH) such as steatosis severity, inflammation, ballooning, and fibrosis (15). Conversely, Baragetti *et al.* found that carriers of the R46L *PCSK9* loss-of-function (LOF) variant display a two-fold increase prevalence of hepatic steatosis [16]. Thus, the direction of the relationship between PCSK9 and NAFLD remains unclear.

To further investigate the clinical robustness of the link between PCSK9 and the severity of NAFLD/NASH, we performed some correlations between plasma PCSK9 levels and both liver fat content assessed by Magnetic Resonance Imaging (MRI) for NAFLD and histological scores for NASH. The patients were recruited from three cohorts and were free of statin therapy to limit some confounding effect [17].

2. Material and methods

2.1 Subjects

Patients included in this study were recruited from three cohorts in two French hospitals (CHU Angers and CHU Dijon). Two populations came from the same University Hospital of Angers. The first, hereinafter referred as Angers' population, gathered patients with non-alcoholic fatty liver disease (NAFLD) recruited in the department of Hepatology in which they underwent liver biopsy to evaluate the severity of their liver lesions; while the second cohort called "Numevox" (ClinicalTrials.gov identifier: NCT00997165) was recruited in the department of Endocrinology where all patients had at least one metabolic syndrome criterion. Fifty patients were common to Angers' and Numevox cohorts, but the data and blood samples were collected at different times. The third cohort, previously described [18], came from a population of patients with type 2 diabetes (T2D) followed in the department of Endocrinology from the University Hospital of Dijon. All patients receiving statin therapy at the time of evaluation were excluded from the analyses. The institutional ethics committee approved all the three studies and written informed consent was obtained from each participant.

For each patient, the following data were collected: demographic (age, gender) and anthropometric (weight, BMI, waist size, systolic and diastolic blood pressure) features. Diabetes status was defined by the use of oral antidiabetic drugs or insulin therapy.

2.2 Blood sampling and PCSK9 ELISA

Venous blood samples were obtained in the morning after an overnight fast for the three cohorts. Standard and common biological analyses included lipid profile (direct measurement of total cholesterol, HDL-C, triglycerides TG, the LDL-C being calculated with Friedewald's formula), liver tests (transaminases, gamma-glutamyl transferase), eGFR and hs-CRP. Additional biochemical metabolic parameters were measured in some, but not all, cohorts: ferritin and adiponectin (Numevox and Dijon); fibroblast growth factor 21 (FGF-21) (Numevox).

Fasting plasma PCSK9 concentrations were assayed in duplicate using a commercially available quantitative sandwich ELISA assay (Circulex CY-8079; CycLex Co, Nagano, Japan) and following the manufacturer's instructions, as previously described [14].

2.3 Non-invasive liver steatosis assessment

In the Dijon cohort, liver fat content (LFC) was measured using MRI with a MAGNETOM Trio Tim (total imaging matrix) 3.0 Tesla whole-body system (Siemens Healthcare GmbH, Erlangen, Germany), as described previously [19]. In a subgroup of patients, LFC was measured using the gold-standard proton magnetic resonance spectroscopy method [20]. In Numevox, an abdominal MRI was performed using a 1.5-T MR system (General Electric Medical Systems, Milwaukee, IL, USA) with a phased-array surface coil. LFC was quantified with a multi-echo gradient-echo (MFGRE) sequence using a previously validated method [21]. LFC calculation was based on the signal intensities measured in two regions of interest in the anterior and posterior right lobe of the liver away from liver vessels.

2.4 Liver histology

Liver biopsy was performed percutaneously (16-gauge Menghini) in the Angers population. Pathological examination was performed by a senior expert specialized in Hepatology and blinded for patient data. Steatosis, lobular inflammation and ballooning were semi-quantitatively evaluated according to the NASH CRN scoring system [22]. The NAFLD activity score (NAS), ranging from 0 to 8, corresponds to the sum of the scores of steatosis, lobular inflammation and ballooning. NASH was defined as the presence of each of the 3 following conditions: steatosis grade ≥1, lobular inflammation grade ≥1, and ballooning grade ≥1. Liver fibrosis was staged from F0 to F4 according to the NASH-CRN scoring system: F0= no fibrosis, F1= perisinusoidal or portal/periportal fibrosis, F2= perisinusoidal and portal/periportal

fibrosis, F3= bridging fibrosis, and F4= cirrhosis. Significant fibrosis was defined as F ≥2 and advanced fibrosis as F ≥3.

2.5 Microarray analysis

In addition to the description of the three populations mentioned above, we present hepatic gene expression data from a fourth source. Human liver samples were collected from 138 overweight individuals visiting the Obesity Clinic at the Antwerp University Hospital, as reported elsewhere [23]. The human study protocol is part of the Hepadip protocol (Belgian registration number B30020071389) and approved by the Ethical Committee of the Antwerp University Hospital (file 6/25/125). Patients were classified on the basis of the NAFLD stage (Grade 1, No NAFLD, Steatosis (S)=0, Ballooning (B)=0, Inflammation (I)=0, Fibrosis (F)=0; Grade 2: NAFLD, S≥1, B or I =0, F≤2; Grade 3: NASH, S≥1 and B≥1 and I≥1, F≤2; Grade 4, NASH + advanced fibrosis, S≥1 and B≥1 and I≥1, and F≥3). Gene expression data were extracted from Affymetrix .CEL files (GEO dataset GSE83452) using GeneSpring v14.3 as described [23].

2.6 Statistical analyses

The characteristics of the three populations were described separately. The association between plasma PCSK9 levels and quantitative parameters was assessed using Spearman's rho coefficient. When studying the association between steatosis severity and PCSK9, we built generalized linear models using standardized plasma PCSK9 levels as explanatory parameter. For multivariate analyses, we adjusted on potential confounding factors involved in metabolic syndrome: age, gender, BMI, TG (Z-score, natural log transformed), HDL-C, LDL-C, systolic blood pressure and diabetes mellitus (binary for Angers and Numevox populations, HbA1C (Z-score, natural log transformed) for Dijon). In addition to this, we verified the expected association between PCSK9 and LDL-C after adjustment on confounding factors using stepwise

backward selection (Supplemental data). Last, mRNA expressions were analyzed using both raw value and Z-score.

A *p*-value inferior to 0.05 was deemed statistically significant. All analyses were performed using R software version 3.5.0 [24].

3. Results

3.1 Baseline characteristics

Baseline characteristics of the three cohorts are shown in **Table 1**. The patients were predominantly male: 61.5% in Angers, 70.5% in Numevox and 53.8% in Dijon. The mean age was similar in Angers and Numevox (53.0 ± 13.1 and 53.3 ± 10.0 years, respectively) but higher in Dijon (58.1 ± 10.1 years). Patients were obese with a mean BMI of 33.0 (± 6.7) $kg.m^{-2}$ in Angers, 31.5 (± 5.5) $kg.m^{-2}$ in Numevox and 34.7 (± 6.5) $kg.m^{-2}$ in Dijon. All patients had T2D in Dijon compared to 32 % in Angers and 24.6% in Numevox.

Liver fat content (LFC) was assessed non-invasively using MRI in two cohorts: Numevox and Dijon with median values of 6.8% and 11.2% respectively. Importantly, LFC measured by the gold-standard magnetic resonance spectroscopy method in a subgroup of patients in Dijon Cohort was similar to MRI derived values (11.0%). Finally, hepatic steatosis defined as LFC > 5.5% [20] was present in 63% and 74% of subjects from Numevox and Dijon, respectively.

3.2 Correlation of plasma PCSK9 levels with demographic and metabolic variables

Plasma median PCSK9 levels were higher in women compared to men consistently in all three cohorts (Angers, median value in women 352 ng/mL *vs.* 276 in men, p=0.04; Numevox, 442 *vs.* 349 ng/mL, p=0.011; Dijon, 303 *vs.* 285 ng/mL, p=0.041). However, no association between diabetes status and PCSK9 level was found in Angers (291 ng/mL for people without diabetes *vs.* 306 ng/mL for people with diabetes, p=0.62) or in Numevox (377 *vs.* 321 ng/mL, p=0.40). As previously reported in the general population [25, 26], circulating PCSK9 levels were positively associated with LDL-C in the three cohorts, although not significantly in Dijon (Angers, Spearman's rho coefficient=0.22, p=0.019; Numevox, rho=0.18, p=0.0055; Dijon, rho=0.16, p=0.074) (**Table 2**). These results were consistent when using parametric approach with standardized PCSK9 level and multivariable regression models adjusted on potential confounding factors (**SupplementalTable 1**). A positive correlation between PCSK9 levels and

both total cholesterol (rho from 0.19 to 0.27) and non-HDL-C (rho from 0.18 to 0.24) was also found to be statistically significant in all three cohorts. Plasma PCSK9 concentration was positively associated with TG in patients with either metabolic syndrome (Numevox: rho=0.18; p=0.006) or T2D (Dijon: rho=0.27; p=0.0015). A weak but significant association was also identified between circulating PCSK9 and hs-CRP in Dijon cohort of patients with T2D (rho=0.18; p=0.037).

Regarding liver enzymes, plasma PCSK9 levels were not correlated with transaminases (AST and ALT) and a positive association between PCSK9 and gamma-glutamyl transferase (GGT) was retrieved in the Numevox cohort only (rho=0.17; p=0.012). In patients with available measurements, a significant and positive association between PCSK9 and alkaline phosphatase (ALP) was found in Angers' cohort (rho=0.21; p=0.022). No association was found between plasma PCSK9 and ferritin, adiponectin or FGF-21 concentrations (**Table 2**).

3.3 Plasma PCSK9 levels and liver fat content

There was no evidence for an association between plasma PCSK9 levels and LFC, assessed by MRI (Numevox, rho=0.06, p=0.54; Dijon, rho=-0.03, p=0.76) or magnetic resonance spectroscopy (Dijon, rho=-0.06, p=0.52) (**Table 2 and Fig. 1**). The parametric approach also failed to show a significant association between standardized PCSK9 levels and percentage of LFC on MRI, including in multivariate analyses (Numevox: β=0.71±1.33, p=0.60; Dijon: β=-1.03±0.90, p=0.25) (**Table 3**).

On liver biopsy performed in Angers' population, standardized PCSK9 levels were not associated with the percentage of steatosis neither in univariate approach (β coefficient, linear regression model =-2.60±2.63, p=0.33), nor in adjusted multivariate model (β=-3.95±2.75, p=0.15) (**Table 4).**

3.4 Plasma PCSK9 and liver histology

The histopathological characteristics of the liver biopsies performed in Angers' cohort (n=122) are shown in **Table 4**. The median of steatosis severity was 25% (10-60%), with 25 patients presenting a steatosis grade 2 (20.5%) and 23 a grade 3 (18.9%). NASH was diagnosed in 62% of patients, NAS ≥ 4 in 47%, significant fibrosis F ≥ 2 in 49% and advanced fibrosis F ≥ 3 in 24%.

There was no significant association between plasma PCSK9 concentrations and severity of inflammation (lobular and portal), ballooning and fibrosis, as well as with NAS score (**Table 4**). Circulating PCSK9 levels were comparable in patients whatever their status for NASH (309 ng/mL (240-389) *vs.* 289 (223-373) in patients without *vs.* with NASH respectively, p=0.54).

3.5 PCSK9 mRNA expression and NAFLD severity

Finally, we investigated the association between hepatic *PCSK9* mRNA expression and NAFLD severity in overweight patients who were prospectively screened for the presence of NAFLD [23]. PPARα and Col1A1 were considered as reference markers, with respectively a negative and positive association with NASH severity and fibrosis. As expected, hepatic *PPAR*α mRNA levels were negatively associated with NAFLD severity (n=138, β estimates for log Z-score and NAFLD=-0.08, p=0.0087), whereas gene expression of *Col1A1*, a marker of liver fibrosis, was positively associated with NAFLD severity (n=137, β estimates for log Z-score and NAFLD=0.10, p=0.00024). However, there was no evidence for an association between *PCSK9* mRNA expression and NAFLD score (n=138, β estimates for log Z-score and NAFLD=0.01, p=0.37) (**Fig. 2**).

Discussion

4.1 Main findings

The major finding of this study is that plasma concentrations of PCSK9 are not associated with the severity of liver steatosis and histological markers of NAFLD/NASH in metabolic high-risk populations. Indeed, there is no significant correlation between circulating PCSK9 levels and LFC assessed non-invasively using the MRI and magnetic resonance spectroscopy techniques in subjects who exhibited mostly significant hepatic steatosis (*i.e.* LFC > 5.5%) or the extent of hepatic steatosis on liver biopsy. Importantly, there is also no correlation between plasma PCSK9 and histological markers of NASH such as portal and lobular inflammation, ballooning and fibrosis. Finally, in a microarray analysis of liver biopsies from overweight patients, *PCSK9* mRNA levels are not associated with severity of NASH.

4.2 Pre-clinical data

These findings are reassuring regarding the safety of PCSK9 inhibitors that are currently used as lipid-lowering therapy in clinical practice [27]. Indeed, several pre-clinical and clinical data have highlighted a potential link between PCSK9 and liver steatosis.

In mice for instance, one study demonstrated that PCSK9 was able to regulate free fatty acid uptake by down-regulating CD36 scavenger receptor expression in hepatocytes, with a subsequent increase in LFC in PCSK9-deficient ($PCSK9^{-/-}$) mice [28]. In addition, it has been shown recently that the transcription factor E2F1 activates the expression of *PCSK9* and that E2F1-deficient mice display a NASH phenotype, which is reversed by the adenoviral re-expression of *PCSK9* in the liver [29]. In line with these results, recent network analyses identify *PCSK9* as a gene associated with *de novo* lipogenesis pathway and hepatocellular carcinoma. Interestingly, the expression of lipogenic genes, such as fatty acid synthase (FASN), was reduced in $PCSK9^{-/-}$ mice [30]. Altogether these data suggest that PCSK9-deficiency could promote hepatic steatosis, at least in mice. The picture is not so clear, however, since liver-specific PCSK9

knockout mice displayed resistance to liver steatosis in another study [31]. The reasons for these discrepancies remain unclear but could be explained by differences in genetic background and diets (high-sucrose *vs* high-cholesterol).

4.3 Clinical data

In humans, we have previously reported a significant positive association between plasma PCSK9 levels and LFC assessed by 1H magnetic resonance spectroscopy in young healthy volunteers under short-term high fructose diet (3.5 g/kg/day), but not under baseline conditions [14]. In the large Dallas Heart Study cohort, LFC assessed by magnetic resonance spectroscopy in 2027 subjects was found to be weakly, but significantly, correlated with circulating PCSK9 concentrations (Spearman's rho=0.13; $p<0.0001$) [25]. It should be noticed, however, that LFC in the Dallas Heart Study [32] was lower than in the Numevox and Dijon cohorts (3.6% vs 6.8% and 11.2%, respectively). Thus, it cannot be excluded that the association between plasma PCSK9 and LFC varies with the severity of hepatic steatosis, with a potential role of PCSK9 in the early stages of hepatic steatosis.

4.4 Genetics

Genetics can also help to address the relationship between PCSK9 and NAFLD, by assessing whether patients with *PCSK9* LOF mutations exhibit more frequently hepatic steatosis than controls. To date, there are very few studies that have performed precise hepatic phenotyping to adequately answer this question. For instance, there was no information regarding hepatic function in the seminal paper of Cohen *et al.* in the ARIC cohort [33]. Very recently, Baragetti *et al.* reported that Italian subjects with p.R46L *PCSK9* LOF variant (n=13) had a two-fold increase prevalence of hepatic steatosis (64.3% vs 35.2% in non-carriers, n=521) [15]. It should be noted that liver steatosis was assessed semi-quantitatively using ultrasound technique in this study, which is not the gold standard method compared to MRI. In contrast,

there was no sign of liver disease in the rare patients with a complete absence of PCSK9. The woman who is a compound heterozygote for two *PCSK9* LOF mutations (PCSK9 Y142X/ΔR97) was described to have normal liver tests, despite having no immunodetectable circulating PCSK9 [34]. In addition, we identified a monoallelic *PCSK9* double LOF mutant (R104C/V114A) with dominant negative effect, leading to profound hypobetalipoproteinemia (LDL-C: 7 mg/dL) and no circulating PCSK9 in a 49-year-old man. A moderate liver steatosis was found at ultrasonography when he was hospitalized for a discovery of diabetes with poor glycemic control (HbA1c: 11.5%) [35]. Upon diabetes care and better glycemic control, the subsequent hepatic ultrasounds were normal without liver steatosis and the liver enzymes stayed in the normal ranges (*B. Cariou, personal observation*). Finally, different genome wide association studies (GWAS) did not identify *PCSK9* as a potential gene involved in NAFLD/NASH pathogenesis [36].

4.5 Comparison with other studies

While we were completing the study, Ruscica *et al.* reported that plasma PCSK9 concentrations were positively and significantly associated with severity of hepatic steatosis in 201 patients coming from a cohort of bariatric surgery (n=76) and hepatology (n=125) departments. In addition, circulating PCSK9 levels were positively correlated with necroinflammatory lesions, ballooning and fibrosis [16]. As shown above, our study did not confirm these results. The reason for this discrepancy is unclear but several hypotheses could be formulated. First, the ELISA kit was not the same between the two studies, but usually the different commercial kits give similar results in the literature. It must be underlined however that the canonical association between plasma PCSK9 and LDL-C (or total cholesterol) was not reproduced in the study of Ruscica *et al.* This observation could be due to the fact that some subjects were under statin therapy, a situation that disrupt the association between PCSK9 and LDL-C [17], but the number was quite small (n=15/201) and PCSK9 concentrations were not altered by statins in this study

[16]. In our study, we chose to exclude the patients with lipid-lowering therapies in order to avoid some confounding factors. Reassuringly, we confirmed the well-described associations between PCSK9 and lipid parameters. This association was also observed in multivariable models (Angers, *p-value*=0.0017 and 0.00055 before and after adjustment, respectively; Numevox, 0.055 and 0.043; and Dijon, 0.036 and 0.074) (Supplementary Table 1). Secondly, it cannot be excluded that the baseline characteristics of the populations were different between the two studies, notably regarding LFC or the severity of NASH. Thirdly, Ruscica *et al.* systematically used log-transformed values to study the associations with plasma PCSK9 levels [16]. However, we confirmed our results when we used log-transformed values for PCSK9 concentrations and the same regression models (*data not shown*). Lastly, we cannot exclude that we failed to highlight some weaker associations because of our limited sample size.

Besides circulating PCSK9 concentrations, we also found no association between hepatic *PCSK9* mRNA levels and the severity of NAFLD/NASH in overweight patients. In order to verify the quality of our microarray analysis, we confirmed significant negative and positive correlations between NAFLD severity score and *PPARα* and *Col1A1* gene expression, respectively [23, 37].

4.6 Conclusions

To summarize, our data suggest that PCSK9 is not a key driver of NAFLD/NASH pathogenesis, at least in advanced stages of the disease in high-risk patients with T2D or metabolic syndrome. The observational design of the study allows us to draw some associations but not causation, regarding the role of PCSK9 in NAFLD. Nevertheless, our data are in accordance with the results of the clinical trials with PCSK9 monoclonal antibodies, evolocumab and alirocumab, that are very reassuring regarding some potential hepatic deleterious effect of blocking the extra-cellular PCSK9 pathway. For instance, there was no difference in change of aminotransferase levels between evolocumab and placebo in the large FOURIER trial [5]. Pooled

analysis of 14 randomized trials from the ODYSSEY program found the same reassuring data regarding alirocumab use and liver tests [38]. At this time, we cannot exclude that PCSK9 can exert some additional specific action through an intra-cellular pathway [39] and additional studies are warranted to decipher the role of hepatic PCSK9 in lipogenic and proliferative pathways [30] as well as the liver safety of siRNA directed against PCSK9 (inclisiran) [40].

Conflicts of interest

B.C. received research funding from Sanofi, Regeneron and Pfizer and honoraria from Amgen, Regeneron and Sanofi outside of this submitted work. The other authors have nothing to disclose.

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Author contributions

BC designed the study. MW performed the data analyses. All the authors participated to the acquisition and/or to the interpretation of data. BC and MW wrote the original draft, and all the authors participated in its revision and the final writing of the manuscript. All authors have approved the final version of the manuscript.

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Figure 1. PCSK9 levels and non-invasive evaluation of the liver fat content.

Correlation between PCSK9 levels and percentage of liver steatosis on MRI imaging (left (A): Numevox cohort, center (B): Dijon) and on magnetic resonance spectroscopy (C) (Dijon center only, right).

Figure 2. Association between NAFLD score and mRNA expression of PCSK9 (up) (a), PPARα (center) (b) and Col1A1 (bottom) (c).

Population of 138 overweight individuals (Obesity Clinic, Antwerp University Hospital). mRNA expression as given by raw value (left column) and log Z-score (right)

Supplemental

Supplementary Table 1. Analysis of the association between PCSK9 and LDL-C in the three populations

of Angers, Dijon and Numevox, before and after adjustment on potential confounding factors

SD: standard deviation

Linear regression models using LDL-C as dependent variable and PCSK9 as explanatory variable. Univariate and multivariate analyses. Candidate confounding variables are age, gender, BMI, TG (Z-score, natural log transformed), HDL-C, systolic blood pressure, diabetes (binary for Angers and NUMEVOX, HbA1C (Z-score, natural log transformed) for Dijon).

Confounding variables obtained after backward stepwise selection, with an alpha threshold=20%:

- Angers: BMI, TG (Z-score, natural log transformed), HDL-C, diabetes
- Dijon: age, BMI, TG (Z-score, natural log transformed), HDL-C

Numevox: age, gender, BMI, diabetes

Table 1. Baseline characteristics of the three cohorts (n=478)

Population	Angers n=122	Numevox n=224	Dijon n=132
Demography & clinic			
Age (years)	53.0 ± 13.1	53.3 ± 10	58.1 ± 10.1
Gender(women)	47 (38.5 %)	66 (29.5 %)	61 (46.2 %)
Weight (kg)	94.8 ± 21.9	89.1 ± 15.5	96.9 ± 18.2
BMI ($kg.m^{-2}$)	33.0 ± 6.7	31.5 ± 5.5	34.7 ± 6.5
Waist size (cm)	113.6 ± 16.1	104.5 ± 13.2	-
Diabetes mellitus (yes)	39 (32 %)	55 (24.6 %)	132 (100 %)
Systolic blood pressure (mmHg)	134 ± 14	129 ± 14	137 ± 17
Diastolic blood pressure (mmHg)	79 ± 10	77 ± 9	78 ± 10
Biology			
Total cholesterol (mmol/L)	5.41 ± 1.29	5.43 ± 1.11	5.11 ± 1.12
LDL-C (mmol/L)	3.44 ± 0.98	3.16 ± 0.97	3.12 ± 0.95
HDL-C (mmol/L)	1.14 ± 0.32	1.52 ± 0.48	1.09 ± 0.28
Non-HDL-C (mmol/L)	4.28 ± 1.24	3.92 ± 1.04	4.03 ± 1.1
Triglycerides (mmol/L)	1.95 ± 1.49	1.78 ± 1.11	2.27 ± 1.61
PCSK9 (ng/mL)	322 ± 133	404 ± 180	321 ± 120
ASAT (IU/L)	36 (29; 50)	na	22 (15; 33)
ALAT (IU/L)	56 (35; 79)	33 (21; 51)	35 (25; 52)
GGT (IU/L)	70 (36; 140)	38 (28; 66)	46 (28; 82)
PAL (IU/L)	78 (61; 92)	na	na
FGF-21 (pg/mL)	na	240 (162; 443)	na
hs-CRP (mg/L)	3.1 (1.4; 7.7)	3 (3; 4)	4.1 (2.2; 7.5)
Serum creatinine (µmol/L)	74 (65; 83.8)	74 (66; 83)	83 (68.5; 98)
Ferritin (µg/L)	na	240 (113.5; 402.8)	168 (93; 353)
Adiponectin (µg/mL)	na	6.3 (4.5; 8.7)	4.2 (2.8; 6.7)
Imaging			
LFC (MRI)	na	6.8 (4.5; 24)	11.2 (5.3; 21.4)
LFC (spectroscopy)	na	na	11.0 (5.9; 21.1)
Hepatic steatosis (MRI)[a] (yes)	na	67/106 (63%)	89/120 (74%)
Hepatic steatosis (spectroscopy)[a] (yes)	na	na	84/110 (76%)

Values are expressed as size (percentage), mean ± standard deviation if Gaussian or median (25^{th} percentile; 75^{th} percentile) in case of skewed distribution

ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; FGF-21: fibroblast growth factor 21; GGT: γ-glutamyltransferase; hs-CRP: high sensitivity C-reactive protein; LFC: liver fat content; MRI: magnetic resonance imaging; na: not available

[a]Defined as LFC > 5.5% on MRI or magnetic resonance spectroscopy, respectively

Table 2. Correlation between PCSK9 levels and clinical, biological and imaging characteristics of the three populations (n=478)

	Angers (n=122)		Numevox (n=224)		Dijon (n=132)	
	Rho (ρ)	***p-value***	**Rho (ρ)**	***p-value***	**Rho (ρ)**	***p*-value**
Demography, clinic						
Age	-0.08	0.35	-0.07	0.32	0.04	0.66
Weight	0.02	0.86	-0.07	0.28	-0.07	0.41
BMI	0.10	0.29	-0.01	0.87	0.03	0.71
Waist size	0.11	0.24	-0.04	0.55	na	na
Systolic blood pressure	-0.07	0.43	-0.07	0.30	0.02	0.79
Diastolic blood pressure	-0.05	0.62	0.02	0.78	-0.08	0.35
Biology						
Total cholesterol	**0.19**	**0.037**	**0.27**	**<0.0001**	**0.23**	**0.007**
LDL-C	**0.22**	**0.019**	**0.18**	**0.0055**	0.16	0.074
HDL-C	0.02	0.85	0.13	0.052	-0.13	0.15
Non-HDL-C	**0.18**	**0.042**	**0.22**	**0.0008**	**0.24**	**0.0055**
Triglycerides	0.10	0.25	**0.18**	**0.006**	**0.27**	**0.0015**
AST	-0.02	0.80	na	na	0.07	0.45
ALT	-0.03	0.77	0.04	0.54	0.0	0.96
GGT	0.05	0.55	**0.17**	**0.012**	0.07	0.43
ALP	**0.21**	**0.022**	na	na	na	na
FGF-21	na	na	0.01	0.87	na	na
hs-CRP	0.11	0.24	0.04	0.57	**0.18**	**0.037**
Serum creatinine	-0.12	0.18	-0.11	0.093	0.09	0.31
Ferritin	na	na	-0.02	0.75	-0.17	0.12
Adiponectin	na	na	0.0	0.98	0.16	0.072
Imaging						
LFC on MRI	na	na	0.06	0.54	-0.03	0.76
LFC on magnetic resonance spectroscopy	na	na	na	na	-0.06	0.52

Spearman's rho coefficients are given. Results with $p < 0.05$ are marked in bold.
ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; FGF-21: fibroblast growth factor 21; GGT: γ-glutamyltransferase; hs-CRP: high sensitivity C-reactive protein; LFC: liver fat content; MRI: magnetic resonance imaging; na: not available

Table 3. Study of the association between steatosis severity and PCSK9, with and without adjustment on potential confounding parameters. Dijon (n=114) and Numevox (n=106) populations

	LFC on MRI (%) Linear regression		Steatosis on MRI[a] (binary) Logistic regression	
	***β* (±SD)**	***p*-value**	**OR (95% CI)**	***p*-value**
Dijon, standardized PCSK9 level (+1 SD)				
Univariate	-0.38 ± 0.86	0.66	0.94 [0.62; 1.42]	0.76
Multivariate[b]	-1.03 ± 0.90	0.25	0.86 [0.53; 1.40]	0.55
Numevox, standardized PCSK9 level (+1 SD)				
Univariate	1.15 ± 1.29	0.37	1.33 [0.86; 2.07]	0.21
Multivariate[b]	0.71 ± 1.33	0.60	1.25 [0.70; 2.22]	0.46

LFC: liver fat content; MRI: magnetic resonance imaging; SD: standard deviation

[a] Defined as LFC >5.5% on MRI

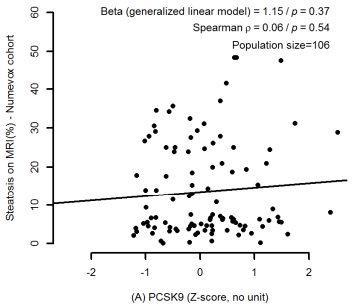
[b] Multivariate models adjusted on age, gender, body mass index, TG (log transformed), HDL-C, LDL-C, systolic blood pressure and:

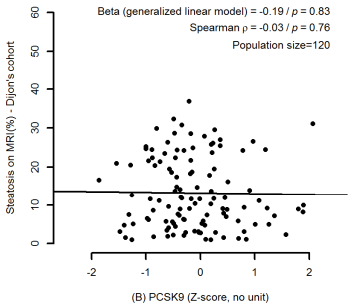
- for Dijon: HbA1C (Z-score, natural log transformed)
- for Numevox: presence of diabetes (yes *vs.* no)

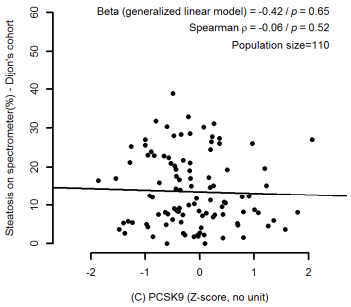
Table 4. Liver lesions on biopsy and association with serum PCSK9 levels, univariate and multivariate analyses. Anger's population (n=122)

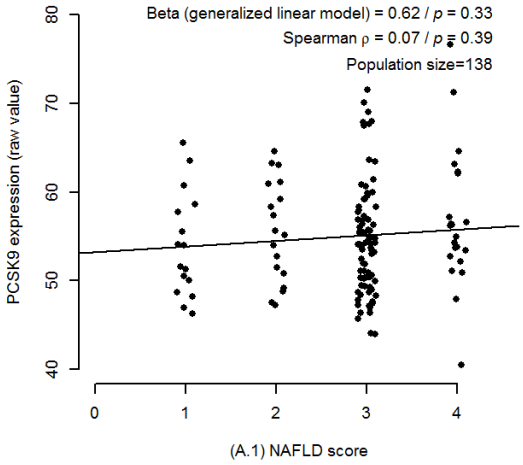
			PCSK9 (Z-score, unit=SD)			
			UNIVARIATE		MULTIVARIATE*	
		n=122	β estimates ± SD	*p*-value	β estimates ± SD	*p*-value
Liver biopsy						
Steatosis severity (%), median=25%, 25th-75th percentile=10-60%		n=117	-2.60 ± 2.63	0.33	-3.95 ± 2.75	0.15
NAS score (grade 0 to 8), median=3, 25th-75th percentile=2-4		n=117	-0.21 ± 0.16	0.21	-0.31 ± 0.17	0.082
Steatosis severity (grade)		n=122	-0.11 ± 0.089	0.22		
	0	20 (16.4 %)				
	1	54 (44.3 %)				
	2	25 (20.5 %)				
	3	23 (18.9 %)				
Lobular inflammation (grade)		n=122	-0.067 ± 0.055	0.22		
	0	33 (27.0 %)				
	1	75 (61.5 %)				
	2	14 (11.5 %)				
Portal inflammation (grade)		n=106	-0.088 ± 0.079	0.27		
	0	36 (34.0 %)				
	1	36 (34.0 %)				
	2	34 (32.1 %)				
Ballooning (grade)		n=122	-0.025 ± 0.065	0.70		
	0	33 (27.0 %)				
	1	60 (49.2 %)				
	2	29 (23.8 %)				
Fibrosis (Kleiner's stage)		n=122	-0.17 ± 0.11	0.12		
	0	21 (17.2 %)				
	1	41 (33.6 %)				
	2	31 (25.4 %)				
	3	20 (16.4 %)				
	4	9 (7.4 %)				

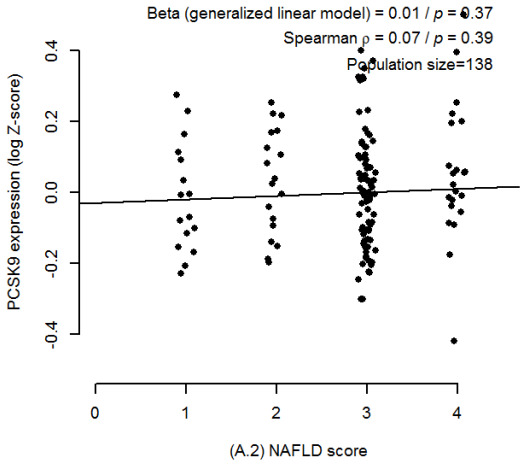
Standardized PCSK9 levels are used.
NAS: NAFLD (Non-Alcoholic Fatty Liver Disease) activity score
* Generalize linear models, multivariate approached adjusted on age, gender, body mass index, triglycerides (Z-score, natural log transformed), HDL-C, LDL-C, systolic blood pressure and diabetes mellitus (yes *vs.* no)

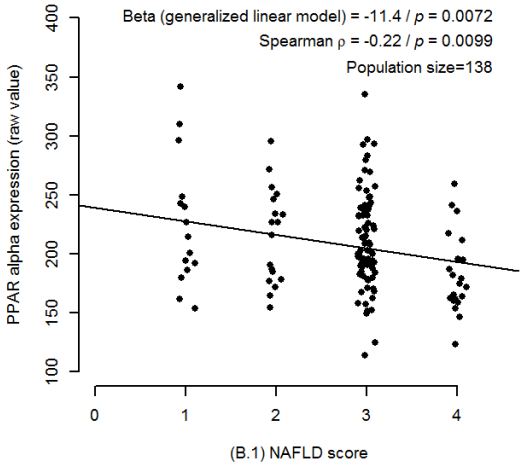


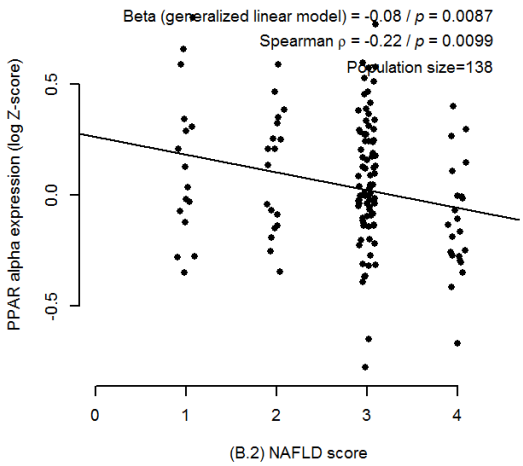


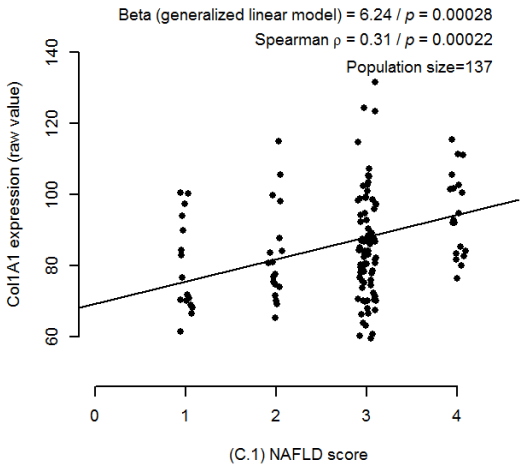


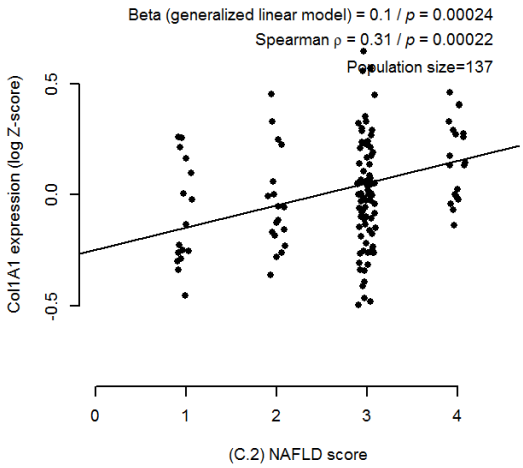

Beta (generalized linear model) = 0.62 / p = 0.33
Spearman ρ = 0.07 / p = 0.39
Population size=138
PCSK9 expression (raw value)
(A.1) NAFLD score


Beta (generalized linear model) = 0.01 / p = 0.37
Spearman ρ = 0.07 / p = 0.39
Population size=138
PCSK9 expression (log Z-score)
(A.2) NAFLD score


Beta (generalized linear model) = -11.4 / p = 0.0072
Spearman ρ = -0.22 / p = 0.0099
Population size=138
PPAR alpha expression (raw value)
(B.1) NAFLD score


Beta (generalized linear model) = -0.08 / p = 0.0087
Spearman ρ = -0.22 / p = 0.0099
Population size=138
PPAR alpha expression (log Z-score)
(B.2) NAFLD score


Beta (generalized linear model) = 6.24 / p = 0.00028
Spearman ρ = 0.31 / p = 0.00022
Population size=137
Col1A1 expression (raw value)
(C.1) NAFLD score


Beta (generalized linear model) = 0.1 / p = 0.00024
Spearman ρ = 0.31 / p = 0.00022
Population size=137
Col1A1 expression (log Z-score)
(C.2) NAFLD score

Highlights

- PCSK9 is a master regulator of cholesterol metabolism
- The link between PCSK9 and liver steatosis is unclear
- Plasma PCSK9 levels are not associated with steatosis severity assessed by MRI
- Hepatic PCSK9 expression is not correlated with severity of NASH
- These results are reassuring regarding the clinical use of PCSK9 inhibitors