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# Plasma proprotein convertase subtilisin kexin type 9 is not altered in subjects with impaired glucose metabolism and type 2 diabetes mellitus, but its relationship with non-HDL cholesterol and apolipoprotein B may be modified by type 2 diabetes mellitus: The CODAM study

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A B S T R A C T

*Objective:* Type 2 diabetes mellitus (T2DM) is associated with elevated plasma apolipoprotein B and triglycerides levels, reduced HDL cholesterol and the presence of small-dense LDL particles. The present study was conducted to investigate the role of plasma proprotein convertase subtilisin kexin type 9 (PCSK9) levels, a regulator of LDL-receptor expression, in the occurrence of diabetic dyslipidemia.
*Methods:* Plasma PCSK9 was measured in a cohort of subjects with normal glucose metabolism (NGM; $n=288$), impaired glucose metabolism (IGM; $n=121$) and type 2 diabetes mellitus (T2DM; $n=139$) to study whether its relation with plasma apolipoprotein B, triglycerides, total cholesterol, non-HDL cholesterol, LDL cholesterol and HDL cholesterol differed by levels of glucose metabolism status.
*Results:* Plasma PCSK9 levels were not different between the three groups (82, 82 and 80 ng/mL in NGM, IGM and T2DM, respectively). PCSK9 was positively associated with total cholesterol, non-HDL cholesterol, LDL cholesterol, apolipoprotein B and triglycerides levels in all subgroups. The regression slopes for the associations with non-HDL cholesterol were steeper among individuals with T2DM than with NGM ($\beta=0.016$ versus $\beta=0.009$, $p$-interaction = 0.05). Similar results were obtained for the relation with apolipoprotein B ($\beta=0.004$ versus $\beta=0.002$, $p$-interaction = 0.09).
*Conclusions:* Although glucose metabolism status *per se* is not associated with plasma PCSK9 levels, the presence of T2DM may modify the relation between plasma PCSK9 and non-HDL cholesterol and apolipoprotein B. These observations should be regarded as hypothesis generating for further studies aimed at elucidating the role of PCSK9 in the pathogenesis and treatment of diabetic dyslipidemia.

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## 1. Introduction

Patients with type 2 diabetes mellitus (T2DM) frequently display a typical dyslipidemia – often referred to as diabetic dyslipidemia – that is characterized by elevated plasma apolipoprotein B and triglycerides levels, reduced HDL cholesterol and the presence of small-dense LDL particles, which is an important determinant of macrovascular complications [1]. Stable isotope studies have demonstrated that VLDL overproduction, at a background of hepatic steatosis and insulin resistance, is one of the pathophysiological hallmarks of diabetic dyslipidemia [2,3]. In addition, there is evidence that the fractional catabolic rate of LDL particles is impaired in patients with T2DM [4,5], which has been attributed to a decreased LDL receptor expression [6]. However, the exact role and regulation of the LDL receptor in relation to diabetic dyslipidemia has not been extensively studied, given the elaborate and expensive nature of stable isotope studies.

Proprotein convertase subtilisin kexin type 9 (PCSK9) is an important regulator of LDL receptor expression. It promotes the degradation of the LDL receptor in hepatocytes by two differ-

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ent pathways, either intracellularly or as a systemically secreted protein that binds the LDL receptor, resulting in a combined internalization and subsequent degradation [7]. A high expression of PCSK9 is therefore positively associated with plasma LDL cholesterol levels [7].

The systemic release of PCSK9 enables its plasma measurement in large cohort studies, which can be used to gain more insight in the effect of PCSK9 on lipoprotein metabolism in specific disease entities, such as T2DM. Indeed, previous epidemiological studies have reported a relation between plasma PCSK9 levels and plasma glucose and insulin, suggesting a role for PCSK9 in T2DM [8,9]. However, these studies did not specifically address whether the role of plasma PCSK9 in relation to plasma lipids differs between individuals with T2DM or at risk for T2DM and those without T2DM.

The aim of this study was therefore to compare plasma PCSK9 levels between subjects with a broad spectrum of glucose tolerance, i.e. normal glucose metabolism (NGM), impaired glucose metabolism (IGM) and T2DM. Given the previously reported positive relation with plasma glucose [8,9], we hypothesized that plasma PCSK9 levels increase as glucose metabolism deteriorates. In addition, we questioned whether the relation between circulating PCSK9 levels and plasma lipid levels differed according to the degree of disturbed glucose metabolism.

## 2. Methods

### 2.1. Subjects and study design

Subjects were participants of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study, an ongoing prospective cohort study in the Netherlands that was originally designed to study the effects of obesity, glucose tolerance, lipid metabolism, lifestyle and genetics on cardiovascular complications [10]. Briefly, between 1999 and 2001 subjects were selected from a large population-based cohort and included if they were of Caucasian ethnicity and aged above 40 years and had at least one of the following: body mass index (BMI) >25 kg/m$^2$, positive family history for T2DM, history of gestational diabetes, use of antihypertensive medication, postprandial glucose >6.0 mmol/L and glucosuria [10]. The original cohort included 574 subjects [10].

All subjects were subsequently asked to stop any lipid-lowering medication two weeks prior to their visit to the University's research unit where blood withdrawals took place; glucose-lowering drugs were stopped on this visit day. Nineteen subjects did notwithstanding continue their lipid-lowering medication. Given the well-documented scattering effects of lipid-lowering therapy on the relation between plasma PCSK9 levels and plasma lipids [11], which was the primary focus of the current study, these subjects were excluded from this study. In addition, levels of plasma PCSK9 were not obtained in 7 subjects. All analyses in the current study were therefore conducted in 548 subjects of whom 288 were classified as having normal glucose metabolism (NGM), 121 as having impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), further referred to as with impaired glucose metabolism (IGM), and 139 as having T2DM, 77 of which were newly diagnosed on the basis of results from an oral glucose tolerance test (OGTT) [12].

All subjects gave written informed consent. This study was approved by the Medical Ethical Committee of the Maastricht University.

### 2.2. Measurements

Height was measured with subjects standing upright against a stadiometer and body weight was measured with electronic weight scales with subjects wearing light indoor clothes without shoes. BMI was calculated as weight divided by height squared. Waist circumference was measured in standing position at the level midway between the lower rib and the spina iliaca anterior superior. Blood pressure was measured twice in supine position and on the right arm after 5 min rest with an oscillometric precision device (Maxi stable 3, Speidel & Keller). Smoking habits as well as the use of medication were assessed by questionnaires.

Blood was collected after an overnight fast in pre-cooled EDTA tubes. After centrifugation at 3000 rpm for 15 min at 4 °C, plasma aliquots were stored at −80 °C. Measurements of fasting plasma triglycerides, total cholesterol, HDL cholesterol, apolipoprotein B, glucose, HbA1c, creatinine, and insulin were done as described previously [10]. LDL cholesterol was calculated with the Friedewald formula [13].

Renal function was calculated by estimated glomerular filtration rate (eGFR, in ml/min per 1.73 m$^2$) using the Modification of Diet in Renal Disease (MDRD) formula ($[186 * (\text{creatinine}/88.4)^{-1.154} * \text{age}^{-0.203}] * 0.742$ if female) [14].

Coronary artery disease was defined as self-reported myocardial infarction, coronary artery bypass surgery, percutaneous coronary intervention or the presence of signs of myocardial infarction (Minnesota codes 1-1 or 1-2) or ischaemia (Minnessota codes 1-3, 1-4, 4-2, 4-3, 5-1, 5-2, 5-3 or 7-1) on a 12-lead electrocardiogram.

### 2.3. PCSK9 ELISA

Plasma PCSK9 levels were measured with the recently described PCSK9 dual monoclonal antibody sandwich ELISA [11], with minor modifications, including the use of a non-His-tagged recombinant PCSK9 standard. The exact epitopes recognized by the antibodies used in the ELISA are not known at this time. Human PCSK9 used as a standard in the ELISA was cloned from a human liver cDNA library with a resulting construct used to generate an HEK293 stable cell line over-expressing PCSK9. The cDNA sequence used did not code for a His-tag. Cells were grown in serum free media, and the secreted PCSK9 protein was purified using an ion-exchange column followed by size-exclusion chromatography. Identity of the protein was confirmed by N-terminal sequencing, and purity was judged to be greater than 95% based on SDS-PAGE followed by Coomassie blue staining. ELISA wells were coated overnight with anti-PCSK9 monoclonal antibody at a concentration of 5 μg/ml. The following day, wells were aspirated, washed three times with assay buffer (50 mM HEPES, pH 7.40, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA), and blocked for 1 h with TBS-casein blocking buffer (Pierce). Next, 100 μl of non-His-tagged recombinant PCSK9 standards (varying concentrations of recombinant protein in assay buffer) were added to the wells as a standard curve. Afterward, serum samples were diluted 1:20 in assay buffer, added to their respective wells, and the ELISA plate was incubated for 2 h at room temperature. Following aspiration, wells were washed three times with assay buffer, and 100 μl of a 1:1000 dilution of conjugate antibody (HRP-labeled anti-PCSK9 monoclonal antibody, 1 mg/ml) were added to the wells for a 1-h incubation at room temperature. Following aspiration, wells were washed three times with TBST. After the last aspiration of TBST, 100 μl of TMB development substrate (Pierce) were added to the wells and allowed to incubate for 30 min at room temperature. The reaction was stopped with an equal volume of 2 N phosphoric acid, and plates were read at 450 nm. SigmaPlot, version 8.0 was used for fitting of the calibration curves. Serum samples were shipped on dry ice and stored at −70 °C prior to analysis. The freeze–thaw stability was excellent with >90% recovery even after four freeze–thaw cycles. The intra assay coefficient of variation (CV%) was 3.9–8.9% [15].

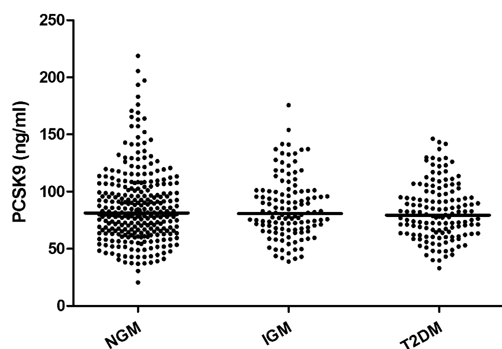


**Fig. 1.** Plasma PCSK9 levels in subjects with NGM, IGM and T2DM. Horizontal bars represent median values for each subgroup.

### 2.4. Statistical analyses

Variables with a skewed distribution, i.e. plasma insulin, triglycerides and PCSK9 were $\log_{10}$ transformed prior to further analyses. Differences in general characteristics between individuals with T2DM or IGM versus with NGM were tested with linear or logistic regression analyses with adjustments for age and sex. Linear regression analyses were used to investigate the associations between levels of PCSK9 and lipids in plasma. These analyses were adjusted for age and sex and were stratified according to glucose metabolism status. Possible difference in the strength of the associations (i.e. the regression slopes) between PCSK9 and plasma lipids between IGM or T2DM versus NGM were appreciated by testing the interaction between glucose metabolism status and PCSK9 in these associations. Interaction terms were considered statistically significant at $p < 0.10$ level (this more liberal cut-off value is more appropriate given the lower sample size of stratified versus whole study population analyses [16]), in concordance with previous studies [17,18]; all other comparisons were considered statistically significant at the conventional $p < 0.05$ level.

All statistical analyses were carried out with the use of the Statistical Package of Social Sciences (SPSS) version 16.0 for Windows (SPSS, Inc., Chicago, IL, USA).

## 3. Results

General characteristics of the study population across categories of glucose metabolism are shown in Table 1. Subjects with T2DM were more often men and older than subjects with NGM. After adjustments for age and sex, subjects with IGM and T2DM were characterized by increasingly higher levels of BMI, waist circumference, blood pressure and use of anti-hypertensive medication, fasting glucose, HbA1c, insulin, triglycerides, lipid-lowering medication and prior CHD, and lower levels of HDL-cholesterol. LDL cholesterol was significantly lower in patients with T2DM but not with IGM when compared with subjects with NGM. Sixty-eight of 139 subjects with T2DM were treated with glucose lowering medication, i.e. metformin ($n = 31$), sulphonylurea derivatives ($n = 50$), acarbose ($n = 1$) and insulin ($n = 13$). Two subjects classified as IGM were treated with a sulphonylurea derivative at the time of their visit to our research unit.

### 3.1. Relation of plasma PCSK9 levels with glucose tolerance

Median (interquartile) levels of plasma PCSK9 were 81.7 ng/ml (65.1–102.7), 81.5 (67.6–100.4) and 79.6 (63.7–98.1) in subjects with NGM, IGM and T2DM, respectively (Fig. 1), which were not significantly different ($p = 0.87$ for IGM versus NGM; $p = 0.49$ for T2DM versus NGM; age- and sex-adjusted). Additional adjustments for waist circumference, plasma insulin levels, eGFR, use of lipid or glucose-lowering medication did not affect these outcomes (data not shown).

Of note, HbA1c levels did not correlate with plasma PCSK9 levels in the overall cohort (regression coefficient beta = 0.008, 95%CI: −0.007 to 0.23, age- and sex-adjusted), nor in any subgroup (data not shown).

### 3.2. Relation of plasma PCSK9 levels with lipid parameters

Plasma PCSK9 levels were significantly associated with total cholesterol, LDL cholesterol, non-HDL cholesterol, triglycerides and apolipoprotein B levels in all glucose metabolism subgroups, after adjustments for age and sex (Table 2). HDL cholesterol was not associated with circulating PCSK9 levels in any group (Table 2).

Of interest, the strength of the associations – depicted by the magnitude of the regression coefficients, in particular for total cholesterol, non-HDL cholesterol, triglycerides and apolipoprotein B, increased as glucose metabolism deteriorated, such that they were stronger among individuals with T2DM than with NGM. Indeed, interaction terms were significant for the relation of PCSK9 with non-HDL cholesterol and for the relation of PCSK9 with apolipoprotein B levels between subjects with NGM and with T2DM ($p = 0.05$ and $p = 0.09$, Fig. 2a and b, respectively). The interaction term for the relation between PCSK9 and triglycerides between NGM and T2DM was borderline significant ($p = 0.10$). None of the associations investigated differed between subjects with IGM and NGM, however. Of note, additional adjustments for use of lipid- or glucose-lowering drugs or, alternatively, re-analysis with subjects naïve to any lipid-lowering medication ($n = 253$ NGM, $n = 106$ T2DM), yielded similar outcomes (data not shown).

## 4. Discussion

The present study was conducted to investigate the role of circulating PCSK9 levels in relation to diabetic dyslipidemia. For this we measured plasma PCSK9 in a well-defined cohort of subjects with different degrees of glucose metabolism. Although the degree of glucose metabolism *per se* was not associated with plasma PCSK9 levels, it did appear to modify the relation between plasma PCSK9 and plasma lipids, in particular non-HDL cholesterol and apolipoprotein B.

The absence of a relation between PCSK9 and the degree of derangement of glucose metabolism is in contrast with previous large cohort studies reporting a significant association between circulating PCSK9 and plasma glucose levels [8,9]. It is unlikely that this discrepancy is due to a lack of power in the current study, since median plasma PCSK9 levels were almost identical between the three groups of interest. Furthermore, HbA1c levels did not relate to plasma PCSK9 levels in the overall cohort. A more likely explanation could be the substantial proportion of subjects with newly diagnosed T2DM (on the basis of results of an OGTT) included in the present study. This may have resulted in a relatively narrow range of HbA1c levels and, consequently, failure to observe any difference in PCSK9 levels between the three groups of interest.

Previous studies have mainly focused on the relation between circulating PCSK9 levels and metabolic and lipid parameters in healthy subjects [8,9,19]. The current report extends these data by demonstrating that these relations, in particular for non-HDL cholesterol and apolipoprotein B, may be modified by T2DM. It should be noted that these interactions were just below the statistical threshold of $p = 0.10$ for interaction and therefore deserve further confirmation in an independent cohort.

Another previously reported factor that modifies the relation between PCSK9 and plasma lipid levels is statin therapy [11]. It is unlikely that the current observations can be accounted for by

**Table 1**
General characteristics of study population.

| | NGM (n = 288) | IGM (n = 121) | T2DM(n = 139) |
|---|---|---|---|
| Men/women, n | 170/118 | 75/46 | 90/49* |
| Age (years) | 58.6 ± 7.4 | 59.9 ± 6.6 | 61.1 ± 6.2* |
| Current smoking (%) | 20 | 18 | 18 |
| BMI (kg/m$^2$) | 27.6 ± 3.9 | 29.0 ± 4.1† | 30.3 ± 4.7‡ |
| Waist circumference (cm) | 96.1 ± 11.1 | 101.1 ± 11.6‡ | 104.8 ± 11.5‡ |
| Systolic blood pressure (mmHg) | 135 ± 18 | 144 ± 20‡ | 148 ± 19‡ |
| Diastolic blood pressure (mmHg) | 80 ± 8 | 84 ± 10‡ | 84 ± 10‡ |
| Antihypertensive medication (%) | 26 | 40† | 56‡ |
| eGFR (ml/min per 1.73 m$^2$) | 84.3 ± 15.5 | 85.1 ± 14.9 | 89.8 ± 20.6‡ |
| Fasting glucose (mmol/L) | 5.3 ± 0.4 | 5.9 ± 0.5‡ | 7.9 ± 1.8‡ |
| HbA1c (%) | 5.6 ± 0.4 | 5.8 ± 0.4† | 6.8 ± 1.1‡ |
| Glucose lowering therapy (%) | 0 | 2 | 49 |
| Insulin (pmol/L) | 59.2 (41.0–68.0) | 67.5 (46.0–100.8)‡ | 86.5 (60–130)‡ |
| Total cholesterol (mmol/L) | 5.2 ± 0.9 | 5.3 ± 0.9 | 5.2 ± 1.1 |
| Non-HDL cholesterol (mmol/L) | 4.0 ± 0.9 | 4.1 ± 0.9 | 4.1 ± 1.2 |
| HDL cholesterol (mmol/L) | 1.3 ± 0.4 | 1.2 ± 0.3† | 1.1 ± 0.3‡ |
| LDL cholesterol (mmol/L) | 3.4 ± 0.9 | 3.4 ± 0.8 | 3.1 ± 0.8† |
| Triglycerides (mmol/L) | 1.2 (0.9–1.6) | 1.7 (1.1–2.2)‡ | 1.8 (1.2–2.4)‡ |
| Apolipoprotein B (g/L) | 1.1 ± 0.2 | 1.2 ± 0.2 | 1.1 ± 0.3 |
| Lipid-lowering medication (%) | 12 | 17 | 24† |
| Coronary heart disease (%) | 18 | 23 | 30† |

Data are expressed as number or frequency (%), mean ± standard deviation or median (interquartile range); all subjects stopped their lipid lowering medication two weeks prior to blood withdrawal (see Section 2). NGM, normal glucose metabolism; IGM, impaired glucose metabolism, T2DM, type 2 diabetes; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* $p < 0.05$, versus NGM.

† $p < 0.05$, versus NGM, after adjustments for age and sex.

‡ $p < 0.001$, versus NGM, after adjustments for age and sex.

**Table 2**
Associations between circulating PCSK9 with plasma lipids stratified by levels of glucose metabolism.

| Dependent variables | NGM (n = 288) | | IGM (n = 121) | | T2DM (n = 139) | |
|---|---|---|---|---|---|---|
| | β | 95%CI | β | 95%CI | β | 95%CI |
| Total cholesterol (mmo/L) | 0.009 | 0.006; 0.013 | 0.010 | 0.004; 0.016 | 0.015 | 0.008; 0.022 |
| LDL cholesterol (mmol/L) | 0.007 | 0.004; 0.011 | 0.006 | 0.001; 0.012 | 0.011 | 0.005; 0.017 |
| HDL cholesterol (mmol/L) | 0.001 | 0.000; 0.002 | −0.001 | −0.003; 0.001 | −0.001 | −0.003; 0.001 |
| Non-HDL cholesterol (mmol/L) | 0.009 | 0.005; 0.012 | 0.011 | 0.004; 0.017 | 0.016 | 0.009; 0.022 |
| $Log_{10}$ triglycerides | 0.001 | 0.000; 0.001 | 0.002 | 0.001; 0.004 | 0.002 | 0.001; 0.003 |
| Apolipoprotein B (g/L) | 0.002 | 0.001; 0.003 | 0.003 | 0.001; 0.004 | 0.004 | 0.002; 0.005 |

*Abbreviations*: β, linear regression coefficient; indicates change in dependent variable per ng/ml increase in PCSK9; CI, confidence interval; NGM, normal glucose metabolism; IGM, impaired glucose metabolism; T2DM, type 2 diabetes mellitus; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
Analyses were adjusted for age and sex.

statin use, since subjects included in the current study were withdrawn from any lipid lowering medication two weeks prior their visit. Furthermore, re-analysis with only subjects who had never been treated with lipid-lowering medication yielded very similar results. Finally, statin therapy *decreases* the strength of the association between PCSK9 and total cholesterol, LDL cholesterol and triglycerides [11] whereas, in the present study, these associations appeared to be *stronger* among individuals with T2DM.

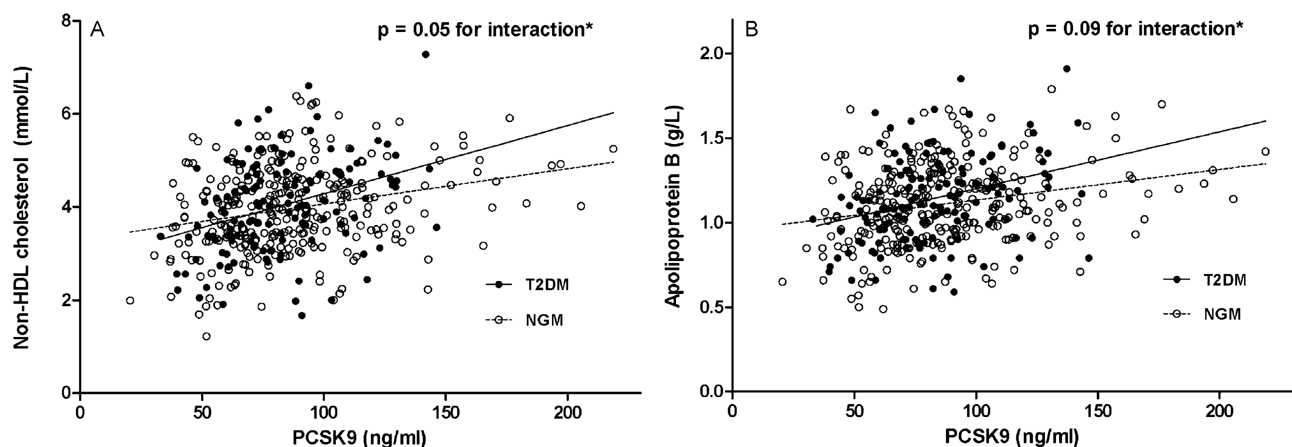


**Fig. 2.** Unadjusted associations between PCSK9 and non-HDL cholesterol (panel A) and apolipoprotein B (panel B) in individuals with NGM and T2DM; *p-values for interaction are age- and sex-adjusted.

The expression of the LDL receptor is not only mediated by PCSK9, but also by several other factors, such as thyroid hormone [20]. Of interest, Bakker and colleagues have shown that the relation between plasma thyrotropin levels and LDL cholesterol is modified by the degree of insulin resistance, i.e. the association of thyrotropin with LDL cholesterol levels is stronger as insulin resistance increases [21]. These observations therefore appear to be in concordance with our current results that T2DM may act as an effect modifier in the relation between PCSK9 and lipoprotein metabolism.

Although this study should be regarded as hypothesis generating and the exact mechanism for this interaction remains to be elucidated, one could speculate on its pathogenesis. For instance, it can be anticipated that VLDL overproduction, as documented in T2DM [2,3], does not have substantial consequences on plasma lipid levels as long as their clearance is not impaired. However, when there is an additional low expression of LDL receptors, due to among others high levels of PCSK9 [7], it can be expected that these defects act synergistically on plasma lipid levels resulting in a steeper slope for the relation between PCSK9 and non-HDL cholesterol/apolipoprotein B, as observed in T2DM. The lack of interaction of LDL cholesterol levels with T2DM in the present study may be explained by the fact that LDL cholesterol levels do not necessarily reflect LDL particle number – which is the actual ligand for the LDL receptor – in particular in the high triglycerides range (>1.5 mmol/L) [22]. Previous studies have shown that apolipoprotein B and non-HDL cholesterol are better indices of LDL particle number than plasma LDL cholesterol levels [22].

Management of diabetic dyslipidemia currently consists of life style modifications, fibrates and statin therapy [23]. Of interest, the latter therapy has consistently been associated with an apparently paradoxical increase in plasma PCSK9 levels, also in T2DM [24,25], whereas conflicting results have been obtained for fibrate therapy [25,26]. These observations suggest that additional treatment with a PCSK9 antagonist, which is under current development [27], could work in a synergistic manner with statin therapy to further reduce plasma lipid levels. Whether patients with T2DM could also benefit from PCSK9 antagonizing therapy needs to be further investigated.

In conclusion, the present study clearly demonstrates that the degree of glucose intolerance is not associated with plasma PCSK9 levels. It may, however, modify the relation between circulating PCSK9 and plasma lipid levels, as reflected by a stronger relation between PCSK9 with non-HDL cholesterol and apolipoprotein B levels in patients with T2DM. These epidemiological observations should be regarded as hypothesis generating for further studies aimed at elucidating the role of PCSK9 in the pathogenesis and treatment of diabetic dyslipidemia.

## Conflict of interest

J.T. and R.K. are employed by Eli Lilly and Company and own stock in Eli Lilly and Company. M.B., M.G., I.F., E.F., C.K., N.S., C.G.S. C.D.S. have nothing to declare.

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