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Original Article

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Differential Effects of DPP-4 Inhibitors, Anagliptin and Sitagliptin, on PCSK9 Levels in Patients with Type 2 Diabetes Mellitus who are Receiving Statin Therapy

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Aim: Proprotein convertase subtilisin/kexin type 9 (PCSK9) degrades the low-density lipoprotein (LDL) receptor, leading to hypercholesterolemia and cardiovascular risk. Treatment with a statin leads to a compensatory increase in circulating PCSK9 level. Anagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, was shown to decrease LDL cholesterol (LDL-C) levels to a greater extent than that by sitagliptin, another DPP-4 inhibitor, in the Randomized Evaluation of Anagliptin versus Sitagliptin On low-density lipoproteiN cholesterol in diabetes (REASON) trial. We investigated PCSK9 concentration in type 2 diabetes mellitus (T2DM) and the impact of treatment with anagliptin or sitagliptin on PCSK9 level as a sub-analysis of the REASON trial.

Methods: PCSK9 concentration was measured at baseline and after 52 weeks of treatment with anagliptin ($n=122$) or sitagliptin ($n=128$) in patients with T2DM who were receiving statin therapy. All of the included patients had been treated with a DPP-4 inhibitor prior to randomization.

Results: Baseline PCSK9 level was positively, but not significantly, correlated with LDL-C and was independently associated with platelet count and level of triglycerides. Concomitant with reduction of LDL-C, but not hemoglobin A1c (HbA1c), by anagliptin, PCSK9 level was significantly increased by treatment with sitagliptin (218 ± 98 vs. 242 ± 115 ng/mL, $P=0.01$), but not anagliptin (233 ± 97 vs. 250 ± 106 ng/mL, $P=0.07$).

Conclusions: PCSK9 level is independently associated with platelet count and level of triglycerides, but not LDL-C, in patients with T2DM. Anagliptin reduces LDL-C level independent of HbA1c control in patients with T2DM who are on statin therapy possibly by suppressing excess statin-mediated PCSK9 induction and subsequent degradation of the LDL receptor.

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Key words: Anagliptin, Sitagliptin, Dipeptidyl peptidase-4 inhibitor, Proprotein convertase subtilisin/kexin

Introduction

Dipeptidyl peptidase-4 (DPP-4) inhibitors, a class of antidiabetic drugs, have distinct structures among the drugs[1)] and include peptidomimetic and non-peptidomimetic agents[2)]. DPP-4 inhibitors are

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also categorized into three classes of binding pocket based on their binding subsites[3, 4]. Therefore, there might be an effect of each drug as well as a class effect of DPP-4 inhibitors. As a possible drug effect, anagliptin, a DPP-4 inhibitor, has been reported to decrease low-density lipoprotein (LDL) cholesterol (LDL-C)[5-8]. In the Randomized Evaluation of Anagliptin versus Sitagliptin On low-density lipoprotein cholesterol in diabetes (REASON) trial, treatment with anagliptin for 52 weeks was associated with a greater reduction in LDL-C levels than was treatment with sitagliptin in patients with type 2 diabetes mellitus at high risk for cardiovascular events and with LDL-C level of >100 mg/dL who were receiving statin therapy[7].

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease synthesized primarily in the liver and has been identified as a key regulator of LDL receptor processing[9]. PCSK9 directly binds to the LDL receptor and subsequently promotes degradation of the LDL receptor[10-12] through an endosomal/lysosomal pathway[13]. Gain-of-function mutations of the gene encoding PCSK9 are associated with hypercholesterolemia[14]. On the other hand, PCSK9 loss-of-function variants decrease LDL-C level, leading to a reduction in coronary artery disease[15-18]. It has recently been reported that PCSK9 inhibitors, evolocumub and alirocumab, significantly decrease LDL-C level and reduce cardiovascular events[19, 20]. Furthermore, emerging experimental and clinical evidence has recently shown that PCSK9 accelerates atherosclerosis and coronary artery disease beyond degradation of the LDL receptor[21, 22], suggesting that the function of PCSK9 is physiologically and clinically significant. Interestingly, circulating PCSK9 concentration is associated with several aspects of lipid and inflammation pathways and severity of coronary artery disease by the Gensini score[23]. Notably, treatment with a statin has been shown to increase PCSK9 level[24] due to a low intracellular cholesterol-mediated compensatory induction of PCSK9 in the liver[25].

However, little is known about the impact of DPP4 inhibitors on PCSK9-mediated cholesterol metabolism. In the present study, we investigated the impact of DPP-4 inhibitors, anagliptin and sitagliptin, on PCSK9 level in patients with type 2 diabetes mellitus at a high risk for cardiovascular events who were receiving statin therapy as a real-world setting with a relatively long-term intervention.

Methods

Study Patients

Study patients were recruited from the REASON trial[7] registered in Clinicaltrials.gov (NCT02330406). The detailed design including criteria of inclusion and exclusion in the REASON trial were previously reported[7, 26]. In brief, the trial was a multicenter, randomized, open-label, parallel-group design that assessed the effects of anagliptin (100 mg, twice daily) and sitagliptin (50 mg once daily) for 52 weeks on reduction in LDL-C in patients with type 2 diabetes mellitus at high risk for cardiovascular events and whose LDL-C levels were >100 mg/dL despite treatment with a statin. In the first report of the REASON trial, anagliptin was reported to decrease LDL-C level to a greater extent than sitagliptin[7].

The REASON trial was conducted in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan. The protocol and consent forms were approved by the institutional review boards in University of the Ryukyus (No. 731) and each participating center. All enrolled patients provided written informed consent prior to randomization. Sub-analysis studies using stored serum samples were planned in the protocol and were conducted according to the decision of the steering committee. The present study was one of the sub-analysis studies, and the effects of anagliptin and sitagliptin on PCSK9 concertation were investigated. Since more than 80% of the patients in the REASON trial had been treated with a DPP-4 inhibitor prior to randomization, only patients who had been treated with a DPP-4 inhibitor were included in the present study. Among 313 patients who were enrolled in and completed the REASON trial, a total of 250 patients treated with anagliptin (n=122, male/female: 70/52) or sitagliptin (n=128, male/female: 75/53) for 52 weeks were included in the present study. Their serum samples were stored at −80℃ until biochemical analyses.

Measurements

Clinical characteristics, including age, sex, body mass index (BMI) calculated as body weight in kilograms divided by height in meters squared, waist circumference, past medical history, smoking status, alcohol consumption and use of concomitant drugs, were evaluated at baseline. BMI and waist circumference were also measured at 52 weeks. Aspartate transaminase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γGTP), blood urea nitrogen, creatinine and fasting glucose were measured

in each participating center at baseline and at 52 weeks. Estimated glomerular filtration rate (eGFR) was calculated from data for serum creatinine, age and sex using the following equation: eGFR (mL/min/1.73 m^2) = 194 × serum creatinine$^{(-1.094)}$ × age$^{(-0.287)}$ × 0.739 (if female)[27]. Hemoglobin A1c (HbA1c) (presented as the National Glycohemoglobin Standardization Program (NGSP) equivalent value), LDL-C (determined by the direct method), total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides and insulin were measured at baseline and at 52 weeks in a core laboratory (SRL Inc., Tokyo, Japan). PCSK9 concentration was measured using a commercially available enzyme-linked immunosorbent assay kit for PCSK9 (R&D Systems, Minneapolis, Minnesota) as described previously[28].

Statistical Analysis

Continuous variables were expressed as means with standard deviation (SD), means with standard error (SE) or medians with interquartile ranges. Categorical variables were expressed as numbers with percentages and were compared between the anagliptin and sitagliptin treatment groups by the chi-squared test or Fisher's exact test. A one-sample *t*-test was used for comparisons of values at baseline and at 52 weeks within each treatment group, and a two-sample *t*-test was used for comparisons between the treatment groups. The correlation between two continuous variables was determined by using Pearson's correlation coefficient. Multivariable linear regression models were used to explore independent parameters of PCSK9 level and change in PCSK9 level. Age, sex and variables with relatively high correlations determined by Pearson's coefficient ($P \leq 0.1$) were incorporated in the multivariable models after consideration of multicollinearity. Treatment group was also incorporated into the model for change in PCSK9 level. The relationships were expressed with unstandardized regression coefficient, SE of regression coefficient and standardized regression coefficient (β). All statistical analyses were performed at an independent data center (Institute for Clinical Effectiveness, Kyoto, Japan) by study statisticians using JMP 13.1 (SAS Institute Inc, Cary, NC) and SAS 9.4 (SAS Institute Inc, Cary, NC). All P values were two-sided, and $P<0.05$ was considered statistically significant.

Results

Characteristics of Patients at Baseline

Baseline characteristics of the patients treated with anagliptin and sitagliptin are shown in **Table 1**. The mean age of the patients was 68 years, and the prevalences of hypertension, coronary artery disease and stroke were 75%, 45% and 14%, respectively. A strong statin and ezetimibe were used as medications for dyslipidemia in 77% and 9% of the patients, respectively. All of the recruited patients had been treated with a DPP-4 inhibitor prior to randomization. There was no significant difference in age, prevalence of habits of smoking and alcohol drinking, diagnosis including hypertension, coronary artery disease and stroke, or medications between the anagliptin and sitagliptin treatment groups **(Table 1)**. There was no significant difference in PCSK9 level at baseline between the anagliptin and sitagliptin groups **(Supplementary Table 1)**.

Changes in Metabolic Parameters from Baseline to 52 Weeks

Treatment with anagliptin for 52 weeks significantly decreased BMI, diastolic blood pressure and levels of eGFR, total cholesterol and LDL-C and increased HbA1c level **(Table 2)**. On the other hand, treatment with sitagliptin for 52 weeks significantly increased AST, total cholesterol, HDL-C, fasting glucose and HbA1c. There were significant differences in the changes in parameters including AST, total cholesterol, LDL-C and HDL-C from baseline to 52 weeks between the anagliptin and sitagliptin groups **(Table 2)**. PCSK9 level was significantly increased by 11.0% (218 ± 98 vs. 242 ± 115 ng/mL, $P=0.01$) by treatment with sitagliptin but was not significantly increased by treatment with anagliptin (233 ± 97 vs. 250 ± 106 ng/mL, $P=0.07$) **(Fig. 1)**. No significant difference in change in PCSK9 level was found between the anagliptin and sitagliptin groups ($P=0.57$).

Correlation and Multivariable Regression Analyses for PCSK9 Level at Baseline

As shown in **Table 3**, PCSK9 level at baseline was positively correlated with platelet count **(Supplementary Fig. 1A)** and levels of triglycerides **(Supplementary Fig. 1B)** and HbA1c **(Supplementary Fig. 1C)**. Similar correlations between PCSK9 level and the parameters were found when male and female subjects were separately analyzed. There was a tendency for positive correlations of PCSK9 level with waist circumference **(Supplementary Fig. 1D)**, red blood cell count **(Supplementary Fig. 1E)** and LDL-C level **(Supplementary Fig. 1F)**. Multivariable linear regression analysis using age, sex and variables with relatively high correlations ($P \leq 0.1$) after consideration of multicollinearity, including waist circumference, counts of red blood cells and platelets

Table 1. Background of the patients with type 2 diabetes mellitus (n=250)

	Total	Anagliptin	Sitagliptin	P
n (M/F)	250 (145/105)	122 (70/52)	128 (75/53)	0.85
Age (years)	68 ± 10	68 ± 10	68 ± 10	0.58
Smoking habit	116 (46)	63 (52)	53 (41)	0.05
Alcohol drinking habit	152 (61)	77 (63)	75 (59)	0.45
Diagnosis				
Hypertension	187 (75)	96 (79)	91 (71)	0.17
Coronary artery disease	113 (45)	57 (47)	56 (44)	0.64
Stroke	36 (14)	21 (17)	15 (12)	0.22
Medication				
Dipeptidyl peptidase-4 inhibitor[a]	250 (100)	122 (100)	128 (100)	-
Biguanide	123 (49)	63 (52)	60 (47)	0.45
Thiazolidinedione	43 (17)	20 (16)	23 (18)	0.74
α glucosidase inhibitor	37 (15)	15 (12)	22 (17)	0.28
Sulfonylurea	64 (26)	37 (30)	27 (21)	0.09
Glinide	6 (2)	5 (4)	1 (0.8)	0.11
Sodium-glucose cotransport 2 inhibitor	28 (11)	16 (13)	12 (9)	0.35
Insulin	15 (6)	7 (6)	8 (6)	0.86
Statin	250 (100)	122 (100)	128 (100)	-
Strong statin[b]	193 (77)	97 (80)	96 (75)	0.40
Ezetimibe	22 (9)	13 (11)	9 (7)	0.31
Fibrate	12 (5)	8 (7)	4 (3)	0.20
Eicosapentaenoic acid	24 (10)	12 (10)	12 (9)	0.90
Angiotensin II receptor blocker	128 (51)	66 (54)	62 (48)	0.37
Angiotensin-converting enzyme inhibitor	18 (7)	10 (8)	8 (6)	0.55
Calcium channel blocker	114 (46)	63 (52)	51 (40)	0.06
β blocker	58 (23)	30 (25)	28 (22)	0.61
Diuretic	39 (16)	21 (17)	18 (14)	0.49
Mineralocorticoid receptor antagonist	11 (4)	5 (4)	6 (5)	0.82
Aspirin	108 (43)	58 (48)	50 (39)	0.18
Ticlopidine	11 (4)	6 (5)	5 (4)	0.70
Other anti-platelet drugs	69 (28)	36 (30)	33 (26)	0.51

Variables are expressed as number (%) or means ± SD.
[a]The use before the study; [b]Indicates atorvastatin, rosuvastatin and pitavastatin.

and levels of LDL-C, triglycerides and HbA1c, demonstrated that platelet count and level of triglycerides were independent predictors of PCSK9 level at baseline (R^2=0.119) **(Table 4)**.

Correlation and Multivariable Linear Regression Analyses for Change in PCSK9 Level

Change in PCSK9 level was negatively correlated with PCSK9 concentration at baseline **(Supplementary Table 2)**. There was a tendency for correlations of change in PCSK9 level with the changes in the parameters of waist circumference, creatinine, eGFR, LDL-C, HDL-C and triglycerides **(Supplementary Table 2)**. Multivariable linear regression analysis using age, sex, treatment group and variables with relatively high correlations ($P \leq 0.1$) after consideration of multicollinearity, including PCSK9 level at baseline and changes in eGFR, LDL-C and triglycerides, demonstrated that only basal PCSK9 level was an independent predictor of change in PCSK9 level **(Supplementary Table 3)**.

Discussion

The present study demonstrated that PCSK9 level at baseline is independently associated with platelet count and level of triglycerides, but not LDL-C, in patients with type 2 diabetes mellitus. Furthermore, concomitant with a reduction of LDL-C by treatment with anagliptin, PCSK9 level tended to be increased, but not significantly, in the anagliptin-treated patients with type 2 diabetes mellitus,

Table 2. Characteristics of the patients treated with sitagliptin or anagliptin for 54 weeks

	Anagliptin ($n=122$)			Sitagliptin ($n=128$)			P^a
	Baseline	52 weeks	P	Baseline	52 weeks	P	
Body mass index	26.9 ± 3.8	26.6 ± 4.1	0.02	25.8 ± 3.7	25.8 ± 3.9	0.95	0.11
Waist circumference (cm)	94.5 ± 11.1	93.9 ± 11.0	0.27	92.8 ± 10.1	92.4 ± 9.9	0.29	0.89
Systolic blood pressure	134 ± 16	132 ± 13	0.16	132 ± 16	133 ± 14	0.53	0.14
Diastolic blood pressure	73 ± 12	70 ± 11	0.02	71 ± 11	71 ± 12	0.93	0.09
White blood cell (x 10^2/μL)	6.5 ± 1.6	6.5 ± 1.7	0.81	6.1 ± 1.6	6.2 ± 1.6	0.28	0.36
Red blood cell (x 10^4/μL)	461 ± 51	460 ± 55	0.73	454 ± 44	455 ± 50	0.71	0.61
Platelet (x 10^4/μL)	22.1 ± 6.2	21.8 ± 6.4	0.33	21.4 ± 5.1	21.4 ± 5.1	0.86	0.47
AST (IU/L)	23 (18 - 31)	23 (18 - 30)	0.24	21 (18 - 27)	20 (18 - 25)	0.01	0.01
ALT (IU/L)	22 (15 - 34)	21 (14 - 35)	0.36	19 (14 - 26)	18 (15 - 25)	0.35	0.19
γGTP (IU/L)	31 (18 - 49)	28 (19 - 43)	0.14	24 (18 - 36)	24 (18 - 35)	0.13	0.08
Blood urea nitrogen (mg/dL)	16.9 ± 6.0	17.0 ± 5.4	0.81	17.0 ± 5.9	17.3 ± 5.6	0.45	0.69
Creatinine (mg/dL)	0.85 ± 0.28	0.87 ± 0.29	0.07	0.87 ± 0.30	0.88 ± 0.30	0.48	0.25
eGFR (mL/min/1.73 m^2)	67.1 ± 19.8	64.8 ± 19.3	0.02	66.0 ± 18.4	65.1 ± 18.5	0.24	0.24
Total cholesterol (mg/dL)	190 ± 30	185 ± 26	0.01	185 ± 29	189 ± 25	0.049	< 0.01
LDL cholesterol (mg/dL)	111 ± 21	106 ± 20	< 0.01	109 ± 23	111 ± 20	0.41	0.01
HDL cholesterol (mg/dL)	53 ± 14	53 ± 13	0.52	54 ± 12	55 ± 12	0.01	0.03
Triglycerides (mg/dL)	142 (102 - 195)	138 (97 - 201)	0.87	112 (82 - 157)	115 (82 - 160)	0.62	0.65
Fasting glucose (mg/dL)	142 ± 42	148 ± 51	0.08	137 ± 34	144 ± 39	< 0.01	0.81
Insulin (μU/mL)	8.1 (5.8 - 14.3)	9.0 (5.4 - 14.0)	0.47	6.9 (4.7 - 11.3)	7.2 (4.5 - 11.2)	0.43	0.67
HbA1c (%)	7.0 ± 0.8	7.1 ± 1.0	0.01	6.8 ± 0.6	7.1 ± 0.9	< 0.01	0.43

Variables are expressed as means ± SD or medians (interquartile ranges).
AST, aspartate transaminase; ALT, alanine transaminase; eGFR, estimated glomerular filtration rate; γGTP, γ-glutamyl transpeptidase; HbA1c, hemoglobin A1c.
[a] For group difference in absolute change from baseline to 52 weeks.

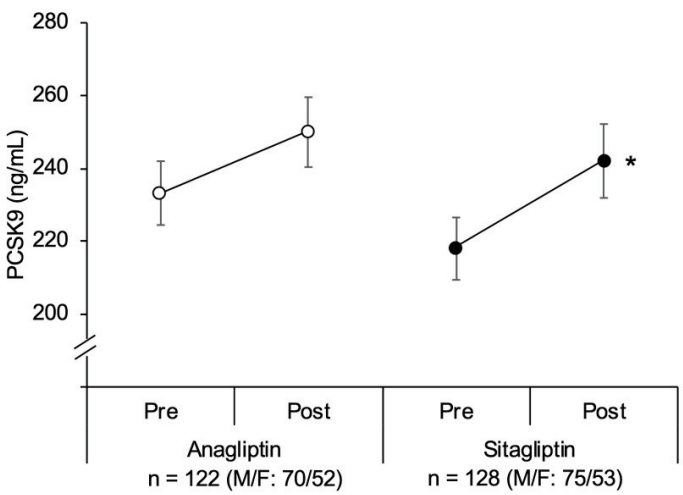

Fig. 1. Effects of anagliptin and sitagliptin on PCSK9 level

Concentrations of proprotein convertase subtilisin/kexin type 9 (PCSK9) at baseline and at 52 weeks in patients treated with anagliptin ($n=122$, male/female: 70/52) and sitagliptin ($n=128$, male/female: 75/53). Values are shown as means ± SE. *$P<0.05$.

Table 3. Correlation analysis for PCSK9 level at baseline ($n=250$)

	Total ($n=250$)		Male ($n=145$)		Female ($n=105$)	
	r	*P*	r	*P*	r	*P*
Age	-0.09	0.14	-0.11	0.18	-0.07	0.45
Body mass index	0.06	0.37	0.03	0.76	0.11	0.27
Waist circumference	0.12	0.052	0.13	0.11	0.11	0.27
Systolic blood pressure	-0.08	0.19	-0.09	0.30	-0.08	0.42
Diastolic blood pressure	-0.05	0.43	-0.11	0.18	0.08	0.43
White blood cell	0.01	0.93	-0.04	0.66	0.08	0.43
Red blood cell	0.11	0.07	0.09	0.30	0.19	0.053
Platelet	0.22	<0.01	0.19	0.02	0.28	<0.01
AST	0.09	0.18	0.13	0.13	0.01	0.89
ALT	0.10	0.11	0.12	0.14	0.07	0.47
γGTP	0.07	0.25	0.06	0.45	0.09	0.35
Blood urea nitrogen	-0.03	0.64	-0.07	0.43	0.03	0.80
Creatinine	-0.01	0.91	-0.01	0.92	0.00	0.97
eGFR	0.02	0.74	0.02	0.81	0.03	0.80
Total cholesterol	0.09	0.14	0.07	0.38	0.12	0.21
LDL cholesterol	0.11	0.08	0.11	0.18	0.11	0.25
HDL cholesterol	-0.06	0.35	-0.09	0.26	-0.03	0.79
Triglycerides	0.18	<0.01	0.16	0.06	0.22	0.03
Fasting glucose	0.05	0.41	0.03	0.76	0.10	0.30
Insulin	0.03	0.68	0.00	0.99	0.16	0.11
HbA1c	0.13	0.04	0.08	0.35	0.22	0.02

Δ, change calculated as parameter in 52 weeks minus that in baseline.
AST, aspartate transaminase; ALT, alanine transaminase; eGFR, estimated glomerular filtration rate; γGTP, γ-glutamyl transpeptidase; HbA1c, hemoglobin A1c.

Table 4. Multivariable regression analysis for PCSK9 level at baseline

	Regression coefficient	SE	Standardized regression coefficient (β)	*P*
Age	-0.19	0.70	-0.02	0.78
Sex (Male)	3.03	13.96	0.02	0.83
Waist circumference	0.93	0.58	0.10	0.11
Red blood cell	0.17	0.14	0.08	0.24
Platelet	4.01	1.12	0.23	<0.01
LDL cholesterol	0.33	0.29	0.07	0.27
Triglycerides	0.19	0.08	0.15	0.02
Hemoglobin A1c	10.53	8.72	0.08	0.23

$R^2=0.119$

dyslipidemia and existing atherosclerotic vascular lesions for which statins were prescribed. On the other hand, treatment with sitagliptin did not change LDL-C but significantly increased PCSK9 level. Neither anagliptin nor sitagliptin improved HbA1c in patients who had been treated with a DPP4 inhibitor, probably due to the limited durability of oral antidiabetic drugs[29]. These findings suggest that anagliptin reduces LDL-C level independent of HbA1c control in patients with type 2 diabetes mellitus who are receiving statin therapy, at least in part, by suppressing excess statin-mediated PCSK9 induction and subsequent degradation of the LDL receptor.

As possible mechanisms of LDL-C reduction by anagliptin, it has been shown in experimental models that anagliptin reduces cholesterol synthesis down-regulated by sterol regulatory element-binding protein

2 (SREBP2) in the liver[30] and inhibits absorption of cholesterol in the small intestine[31]. In human studies, it has been shown that inhibition of cholesterol synthesis[32] and suppression of excess cholesterol synthesis[33] are possible mechanisms for LDL-C reduction by anagliptin. On the other hand, the precise mechanisms by which anagliptin, but not sitagliptin, suppresses an excess increase in circulating PCSK9 level in patients receiving statin therapy are unclear. It has been reported that circulating PCSK9 has a diurnal rhythm synchronous with cholesterol synthesis marker lathosterol[34]. Inhibition of cholesterol synthesis[32] and suppression of excess cholesterol synthesis[33] by anagliptin may be linked to reduced PCSK9 levels. In addition, statin therapy is significantly associated with a compensatory increase in plasma PCSK9 concentration[24]. Up-regulation of PCSK9 by a statin has been shown to occur through a mechanism involving the SREBP2 transcription factor[25]. Anagliptin may reduce PCSK9 level by downregulation of SREBP2 as previously reported in an experimental model[30].

As other mechanisms for the suppression of statin-mediated PCSK9 induction by DPP-4 inhibitors, there are a few possibilities. It has recently been reported that DPP-4 is one of the adipocyte-derived bioactive molecules known as adipokines, though the receptor for soluble DPP-4 remains obscure[35]. Exogenous DPP-4 increased inflammatory reaction and decreased insulin signaling in adipocytes, skeletal muscle cells, and smooth muscle cells, which were rescued by a DPP-4 inhibitor[35-37]. Pharmacological inhibition of the activity of soluble DPP-4 may directly decrease the expression of PCSK9 in hepatocytes. In addition, inflammatory pathways are implicated in mediating the effects of PCSK9 on vascular biology[25]. Inflammation stimulates the expression of PCSK9 [38], whereas knockdown of PCSK9 mediated by small-interfering RNA attenuates the expression of proinflammatory genes[39]. DPP-4 inhibitors have been shown to decrease several inflammatory cytokines and adipokines including tumor necrosis factor-α[40, 41] and fatty acid-binding protein 4 [36, 42], suggesting an additional mechanism by which DPP-4 inhibitors reduce PCSK9 level as a pleiotropic effect. Anagliptin may be able to suppress the increase of PCSK9 concentrations by statins to a greater extent than sitagliptin in patients with type 2 diabetes mellitus and dyslipidemia who are receiving statin therapy, though there has been no direct comparison of the effects of DPP-4 inhibitors on PCSK9 levels. Since inflammatory markers were not investigated in the present study, a distinct mechanism of the suppression of excess PCSK9 induction under the condition of statin treatment by anagliptin needs to be addressed in the future.

In the present study, basal PCSK9 level was positively, but not significantly, correlated with LDL-C level ($r=0.11$, $P=0.08$) **(Table 3)**. It was previously reported that the association between PCSK9 and LDL-C is weak[43-50]. The relatively modest correlation between circulating levels of PCSK9 and LDL-C suggests that circulating PCSK9 level provides a limited indication of PCSK9 activity for mediating degradation of the LDL receptor. Notably, it has been reported that two forms of PCSK9, mature and furin-cleaved PCSK9, circulate in blood[51, 52]. It has also been reported that PCSK9 level is widely associated with several metabolic determinants[28, 43, 45, 50]. In the present study, PCSK9 level was shown to be positively correlated with waist circumference and levels of triglycerides and HbA1c, and there was an independent association between levels of PCSK9 and triglycerides **(Table 4)**, as previously reported[53]. It has recently been reported that PCSK9 induces degradation of CD36, a membrane transporter of long-chain fatty acids, and affects long-chain fatty acid uptake and triglyceride metabolism in adipocytes and in the liver[54]. Furthermore, hepatic PCSK9 expression has been reported to be regulated by sterol regulatory element-binding protein 1c, a key transcription factor that activates transcription of genes involved with fatty acid and triglyceride synthesis[55]. PCSK9 may paly roles of lipid metabolism regulation including not only LDL-C but also triglycerides. PCSK9 inhibitors have been reported to significantly decrease triglycerides level as well as LDL-C level in patients with dyslipidemia[56].

It has been reported that circulating PCSK9 level is positively correlated with platelet count in patients with stable coronary artery disease[57], which was confirmed in the present study using patients with type 2 diabetes and dyslipidemia. PCSK9 level has also been shown to be associated with platelet reactivity[58] and urinary excretion of 11-dehydro-thromboxane-B2 as an unbiased marker of *in vivo* platelet activation[59]. These findings suggest a potential link among a high PCSK9 level, platelet count, atherosclerosis and metabolic disorders. However, direct evidence of a role of PCSK9 in platelet function is still lacking, and interventional trials need to be performed to clarify whether modulation of PCSK9 might also affect platelet function.

The present study has several limitations. First, no washout of DPP4 inhibitors before the beginning of the trial was performed. Most of the study patients

had also been treated with several drugs at baseline. Pretreatment with those drugs may have affected basal PCSK9 concentration and may have modulated the change in PCSK9 level. Second, a total sample size of 300 was estimated to be needed for the original REASON trial[26], and 313 patients were enrolled in and completed the trial[7]. Since the present study as a subanalysis included only 250 patients (anagliptin/sitagliptin: 122/128) by exclusion of patients without pretreatment with a DPP-4 inhibitor, the effect of a DPP-4 inhibitor on PCSK9 level needs to be confirmed using a large number of patients with and without pretreatment with a DPP-4 inhibitor in the future. Third, the present study lacked a placebo control group. Interventional studies using larger number of subjects and a placebo-control design are necessary for determining the impact of DPP-4 inhibitor treatment on circulating PCSK9 level and the relationship between change in PCSK9 level and clinical benefit of DPP-4 inhibitors. Lastly, because the recruited subjects were only Japanese people, it is unclear whether the present findings can be generalized to other ethnicities.

In conclusion, PCSK9 level is independently associated with platelet count and level of triglycerides in patients with type 2 diabetes mellitus. Anagliptin reduces LDL-C levels independent of HbA1c control in patients with type 2 diabetes mellitus at a high risk for cardiovascular events who are receiving statin therapy possibly by suppressing an excess statin-mediated compensatory induction of PCSK9 and subsequent degradation of the LDL receptor. Suppression of excess increase in PCSK9 level by a statin therapy might be beneficial for patients with metabolic and cardiovascular diseases as a pleiotropic effect of anagliptin. A further understanding of drug-induced modulation of PCSK9 will enable the development of new therapeutic strategies for cardiovascular and metabolic diseases.

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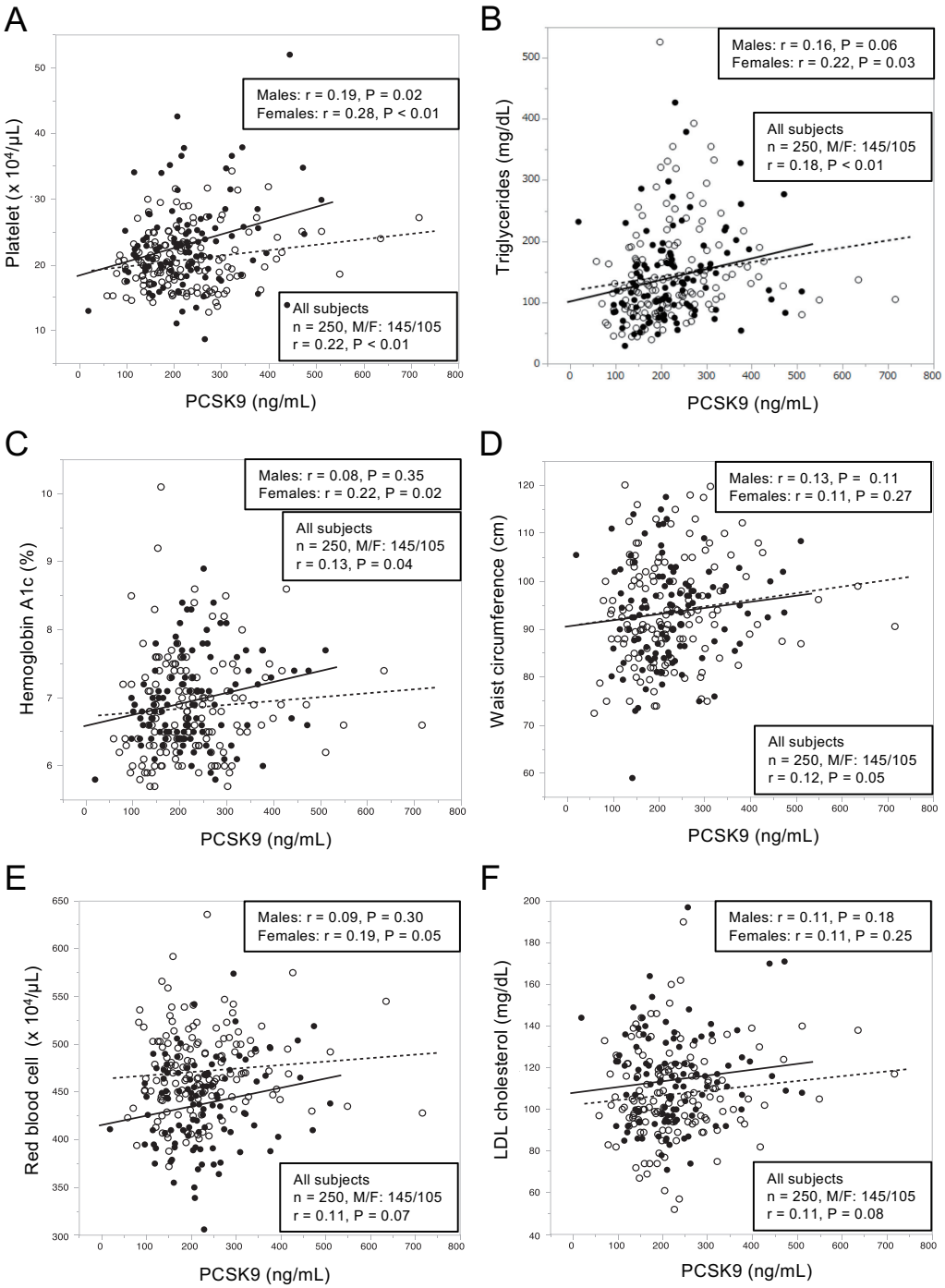
Supplementary Table 1. Characteristics of the patients treated with sitagliptin or anagliptin at baseline

	Anagliptin	Sitagliptin	*P*
n (M/F)	122 (70/52)	128 (75/53)	0.85
Body mass index	26.9 ± 3.8	25.8 ± 3.7	0.03
Waist circumference (cm)	94.5 ± 11.1	92.8 ± 10.1	0.20
Systolic blood pressure	134 ± 16	132 ± 16	0.30
Diastolic blood pressure	73 ± 12	71 ± 11	0.35
White blood cell (x 10^2/μL)	6.5 ± 1.6	6.1 ± 1.6	0.04
Red blood cell (x 10^4/μL)	461 ± 51	454 ± 44	0.27
Platelet (x 10^4/μL)	22.1 ± 6.2	21.4 ± 5.1	0.33
AST (IU/L)	23 (18 - 31)	21 (18 - 27)	0.01
ALT (IU/L)	22 (15 - 34)	19 (14 - 26)	< 0.01
γGTP (IU/L)	31 (18 - 49)	24 (18 - 36)	0.01
Blood urea nitrogen (mg/dL)	16.9 ± 6.0	17.0 ± 5.9	0.90
Creatinine (mg/dL)	0.85 ± 0.28	0.87 ± 0.30	0.57
eGFR (mL/min/1.73 m^2)	67.1 ± 19.8	66.0 ± 18.4	0.65
Total cholesterol (mg/dL)	190 ± 30	185 ± 29	0.19
LDL cholesterol (mg/dL)	111 ± 21	109 ± 23	0.53
HDL cholesterol (mg/dL)	53 ± 14	54 ± 12	0.62
Triglycerides (mg/dL)	142 (102 - 195)	112 (82 - 157)	< 0.01
Fasting glucose (mg/dL)	142 ± 42	137 ± 34	0.28
Insulin (μU/mL)	8.1 (5.8 - 14.3)	6.9 (4.7 - 11.3)	0.07
HbA1c (%)	7.0 ± 0.8	6.8 ± 0.6	0.13
PCSK9 (ng/mL)	233 ± 97	218 ± 98	0.22

Variables are expressed as means ± SD or medians (interquartile ranges).
AST, aspartate transaminase; ALT, alanine transaminase; eGFR, estimated glomerular filtration rate; γGTP, γ-glutamyl transpeptidase; HbA1c, hemoglobin A1c.



Supplementary Fig. 1. Correlations of PCSK9 level with parameters at baseline

A-F. Baseline levels of platelet (A), triglycerides (B) hemoglobin A1c (C), waist circumference (D), red blood cell count (E) and LDL cholesterol (F) were plotted against PCSK9 level at baseline in each subject (n=250). Closed circles and solid regression line: anagliptin treatment group (n=122), open circles and broken regression line: sitagliptin treatment group (n=128).

Supplementary Table 2. Correlation analysis for ΔPCSK9

	Total (n=250)		Anagliptin (n=122)		Sitagliptin (n=128)	
	r	P	r	P	r	P
Age at baseline	0.07	0.26	0.16	0.07	-0.01	0.92
PCSK9 at baseline	-0.39	<0.01	-0.42	<0.01	-0.35	<0.01
ΔBody mass index	-0.03	0.58	-0.09	0.31	0.01	0.90
ΔWaist circumference	0.10	0.13	0.01	0.88	0.18	0.04
ΔSystolic blood pressure	-0.04	0.50	-0.12	0.17	0.02	0.78
ΔDiastolic blood pressure	0.01	0.93	-0.02	0.85	0.02	0.82
ΔWhite blood cell	0.01	0.92	0.07	0.46	-0.06	0.51
ΔRed blood cell	-0.04	0.54	-0.12	0.18	0.04	0.63
ΔPlatelet	-0.02	0.77	0.08	0.37	-0.16	0.08
ΔAST	-0.08	0.23	-0.13	0.16	-0.05	0.59
ΔALT	-0.07	0.30	-0.15	0.10	0.03	0.74
Δγ GTP	0.02	0.74	0.07	0.44	0.01	0.92
ΔBlood urea nitrogen	-0.09	0.17	-0.10	0.26	-0.08	0.39
ΔCreatinine	-0.11	0.09	-0.10	0.29	-0.12	0.17
ΔeGFR	0.10	0.10	0.07	0.43	0.14	0.13
ΔTotal cholesterol	-0.08	0.20	-0.13	0.15	-0.06	0.52
ΔLDL cholesterol	-0.09	0.14	-0.12	0.17	-0.08	0.36
ΔHDL cholesterol	-0.10	0.11	-0.25	0.01	0.04	0.64
ΔTriglycerides	0.11	0.08	0.10	0.26	0.11	0.20
ΔFasting glucose	0.03	0.64	0.03	0.73	0.03	0.76
ΔInsulin	0.06	0.35	0.04	0.63	0.12	0.19
ΔHbA1c	0.03	0.59	0.03	0.78	0.04	0.67

Δ, change calculated as parameter in 52 weeks minus that in baseline.
AST, aspartate transaminase; ALT, alanine transaminase; eGFR, estimated glomerular filtration rate; γGTP, γ- glutamyl transpeptidase; HbA1c, hemoglobin A1c.

Supplementary Table 3. Multivariate regression analysis for ΔPCSK9

	Regression coefficient	SE	Standardized regression coefficient (β)	P
Age	0.34	0.66	0.03	0.61
Sex (Male)	-11.67	12.43	-0.06	0.35
DPP-4i (Sitagliptin)	1.92	12.09	0.01	0.87
PCSK9 at baseline	-0.39	0.06	-0.37	<0.01
ΔeGFR	1.14	0.68	0.10	0.10
ΔLDL cholesterol	-0.46	0.34	-0.08	0.18
ΔTriglycerides	0.16	0.09	0.11	0.07

R^2=0.179
Δ, change calculated as parameter in 52 weeks minus that in baseline.
DPP-4i, Dipeptidyl peptidase-4 inhibitor; eGFR, estimated glomerular filtration rate