河野真理、増田卓、神谷健太郎、高橋由美、見井田和正、山本周平、堀田一樹、木村雅彦、松永篤彦、野田千春、和泉徹	心血管病患者における禁煙宣言の受容は退院後の再喫煙予防において重要な因子となる	心臓リハビリテーション	15	301-05	2010
水谷知泰、和泉徹	慢性心不全	Heart View	14	14-23	2010
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Kameda R, Yamaoka-Tojo M, Wakaume K, Tojo T, Aiba N, Yoshida Y, Machida Y, Matsunaga A, Masuda T, Izumi T.	The change of circulating interleukin-18 are associated with the change of arteriosclerosis, a 5-year observational study from Kitasato Registry of Cardiovascul	第75回日本循環器学会総会・学術集会抄録集	CD-ROM		2011
東條美奈子	BNPを用いた心疾患の二次予防	第75回日本循環器学会総会・学術集会抄録集	CD-ROM		2011

若梅一樹、東條美奈子、根本慎司、亀田 良、饗庭尚子、吉田友紀、町田陽二、増田 卓、和泉 徹	歩数計を利用したセルフモニタリングによる運動指導は身体活動量を増加させ血管内皮機能の改善につながる	日本循環器病予防学会誌 第47回学会抄録集（YIA）	46	137	2011
亀田 良、東條美奈子、若梅一樹、北里梨紗、吉田友紀、東條大輝、町田陽二、饗庭尚子、松永篤彦、増田 卓、和泉 徹	冠動脈疾患及びその高リスク患者において、血中Pentraxin 3（PTX3）濃度はLDL/HDL比と関連する	日本循環器病予防学会誌 第47回学会抄録集	46	155	2011

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CARDIO VASCULAR DIABETOLOGY

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Elevated circulating levels of an incretin hormone, glucagon-like peptide-1, are associated with metabolic components in high-risk patients with cardiovascular disease

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Abstract

Background: Glucagon-like peptide-1 (GLP-1) is an incretin hormone that has a wide range of effects on glucose metabolism and cardiovascular function (e.g., improving insulin sensitivity, reduction in appetite, modulation of heart rate, blood pressure and myocardial contractility). Metabolic syndrome (MetS) is associated with an increased risk of developing atherosclerotic cardiovascular diseases. Novel glycemic control drugs, the dipeptidyl-peptidase-4 (DPP-4) inhibitors, work by inhibiting the inactivation of incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP). In spite of good effects of these drugs in diabetic patients, circulating levels of incretins and their role in MetS are largely unknown.

Methods: To examine relationships between incretin hormones and MetS risk factors, we measured circulating levels of incretins in obese high-risk patients for cardiovascular disease. Fasting serum GLP-1 and GIP levels were measured by ELISA. We performed a cross-sectional analysis of metabolic variables in the fasting state in two subject groups: with MetS (n = 60) and pre-MetS (n = 37).

Results: Fasting levels of Serum GLP -1 in the peripheral circulation were significantly increased correlated with the accumulation of MetS risk factors components ($r = 0.470$, $P < 0.001$). There was a significant interaction between circulating GLP-1 and GIP, serum high-density lipoprotein cholesterol, triglyceride, and serum uric acid concentrations but not waist circumference, fasting glucose, HbA1c, or presence of diabetes.

Conclusion: Circulating levels of GLP-1 in relation to the accumulation in MetS factors suggested that MetS patients with elevated levels of GLP-1 are high-risk patients for cardiovascular disease, independent with the presence of diabetes.

Background

The metabolic syndrome (MetS) is a major public health problem [1] and a multiple risk factor for cardiovascular disease [2,3]. It consists of atherogenic dyslipidemia (elevated triglycerides and low high-density lipoprotein [HDL]), and elevations of blood pressure and glucose, and abdominal obesity with pro-thrombotic and proinflammatory states [1]. MetS is associated with a 5-fold higher risk of developing type 2 diabetes and 2.6-to 3-fold high risk of cardiovascular disease [4,5]. The pathophysiology of MetS is not well defined, and several investigators have sought to identify a unifying factor that could explain all the components of the syndrome. In addition to insulin resistance/hyperinsulinemia, investigators have found several biomarkers to be associated with MetS including leptin [4], catecholamine [6], brain natriuretic peptide (BNP) [7], oxidized low-density lipoprotein cholesterol (LDL)[8], uric acid [9], C-reactive protein (CRP) [4], plasminogen activator inhibitor-1 [4], aldosterone [4], cyctatin C [10], and carboxy-terminal prevasopress in

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(copeptin)[11], highlighting diverse pathophysiological perturbations in MetS [11].

Glucagon-like peptide-1 (GLP-1) is a hormone derived from the prepro-glucagon molecule and is secreted by intestinal L cells [12]. It is the most potent stimulator of glucose-induced insulin secretion and also suppresses *in vivo* acid secretion by gastric glands. Intracerebroventricular GLP-1 is powerfully inhibited in fasting rats [13]. In addition, injection of a specific antagonist of GLP-1 blocked the inhibitory effect of GLP-1 on food intake. GLP-1 receptor is expressed in the central nervous system or in the stomach except β cells of the pancreas, and its every function such as inhibition of insulin secretion, appetite suppression and gastric motor inhibition has a hypoglycemic effect. Thus, the development of GLP-1-releated study as a diabetic drug (e.g., GLP-1 analogs and the dipeptidyl-peptidase-4 [DPP-4] inhibitors) is progressing.

We therefore hypothesized that circulating GLP-1 would be associated with insulin resistance/hyperinsulinemia and MetS. To examine relationships between incretin hormones and MetS components, we measured circulating levels of incretins, GLP-1 and gastric inhibitory polypeptide (GIP), in high-risk patients for cardiovascular disease.

Methods

Subjects

The study included 97 Japanese high-risk outpatients for cardiovascular disease with abdominal obesity (40% female) in the Obesity/Metabolic Syndrome Clinic, Department of Cardioangiology, Kitasato University Hospital. The only exclusionary criterion at enrolment was the treatment with antidiabetic drugs including insulin and oral agents. All subjects gave informed consent before participating in this study, and the ethics committee of the Kitasato University Hospital approved the study design. Body mass index (BMI) was calculated as weight divided by height squared. Systolic and diastolic blood pressure was measured after a rest for at least 15 minutes with a sphygmomanometer in sitting position. Homeostasis model assessment (HOMA-IR) was used as a measure of insulin resistance and was calculated as fasting plasma insulin (μU/mL) × glucose (mg/dL)/405 [14].

Metabolic scores were calculated using MetS components according to the Japanese MetS criteria [15]. The score consisted of four independent components as abdominal obesity, defined as a waist circumference ≥85 cm in men or ≥90 cm in women, hypertriglyceridemia and/or low HDL-cholesterolemia, hypertension, and elevated fasting glucose. The diagnosis of hypertension was established based on blood pressure levels measured at the study visit (≥130/85 mmHg) or a prior diagnosis of hypertension and current treatment with antihypertensive medications. Diabetes/impaired fasting glucose (IFG) was considered present if the subject had history of diabetes or had a fasting glucose level of 110 mg/dL or greater.

Measurement of clinical biomarkers

Blood samples were collected by venipuncture after an overnight fast. Serum was centrifuged (1500 g for 15 min at 4°C) and stored at 4°C until measurement within couple days for biochemical markers, such as triglyceride, LDL cholesterol, HDL cholesterol, insulin, plasma glucose, glycated haemoglobin (HbA1c), uric acid, CRP, and BNP were measured enzymatically in the clinical laboratory of Kitasato University Hospital.

Measurement of incretin hormones

Circulating levels of human GLP-1 and GIP were determined by enzyme-linked immunosorbent assay (ELISA) using the GLP-1 (7-36) amide/prepro-glucagon (98-127) amide enzyme immuno assay EIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) and the human GIP assay kit (Immuno-Biological Laboratories Co., Ltd., Takasaki, Japan), respectively.

Statistical analysis

Continuous data were summarized as either mean ± SD or median and quartiles, and categorical data were expressed as percentages. Data were compared by unpaired *t*-test or Mann-Whitney *U*-tests where appropriate. Differences in proportions of variables were determined by chi-squared analysis. To evaluate the correlations between GLP-1 and selected variables, we calculated Spearman correlation coefficients between circulating levels of fasting GLP-1 and the following variables: 1)conventional risk factors for cardiovascular disease (LDL cholesterol, HDL cholesterol, triglyceride, HbA1c, presence of hypertension, and current smoking), and history of coronary artery disease; 2) measures of adiposity and insulin resistance (BMI, waist circumference, fasting blood glucose, insulin level, and HOMA-IR); and 3) metabolic risk scores (abdominal obesity, hypertension, high triglyceride and/or low HDL cholesterol, and glucose intolerance or diabetes) and MetS-related co-morbidity (hyper uric academia, fatty liver disease, chronic kidney disease, and sleep apnea syndrome). Participants having low metabolic scores (0, 1, or 2) was termed the pre-MetS whereas patients having high metabolic scores (3 or 4) were defined as MetS.

To evaluate the association of GLP-1 with MetS, we constructed multivariable logistic regression models to assess whether circulating GLP-1 was independently associated with MetS. We calculated the odds ratio for the presence of MetS in quartiles of GLP-1 with participants in the lowest quartile of GLP-1 considered the referent group. Adjustments were performed for age and

sex; and age, sex, and beta-blocker use. Two-sided *P*-values < 0.05 were considered significant.

Results

Clinical characteristics and metabolic variables

Sixty patients of MetS and 37 patients of pre-MetS with atherosclerosis-prone conditions participated in the study. The patients with MetS were older than pre-MetS patients but the proportion of women was not different between the groups (Table 1). In MetS patients, BMI was greater than in pre-MetS patients (average BMI 32.1 *vs.* 29.0, $P = 0.015$). Thirty-seven of patients (62%) with MetS were hypertension and used one or more antihypertensive drugs. A half of the patients with MetS were treated with calcium-channel blockers. Twelve patients with MetS and three patients with pre-MetS were diagnosed IFG or type 2 diabetes. None of patients received antidiabetic drugs including insulin. Fasting plasma glucose levels were higher in patients with pre-MetS than with MetS ($P = 0.025$). Predictably, HDL cholesterol was lower in the patients with MetS compared with pre-MetS ($P = 0.001$). Thirty-eight of the patients with MetS were diagnosed dyslipidemia and 45% of the patients received statins whereas only 19% of the patients with pre-MetS used statins ($P = 0.016$). The proportion of participants who have previous and/or current coronary artery disease was sig-

Table 1: Participant characteristics (n = 97).

	MetS (n = 60)	Pre-MetS (n = 37)	*P*-value
Age (year)	52.8 ± 14.2	50.3 ± 16.6	0.494
Sex, female (%)	22 (36.7%)	17 (45.9%)	0.333
BMI (kg/m^2)	32.1 ± 6.5	29.0 ± 4.9	0.015
Waist circumference (cm)	103 ± 15	98 ± 11	0.093
Plasma glucose (mg/dL)	114 ± 28	130 ± 41	0.025
Plasma insulin (IU/mL)	33.0 ± 36.4	33.4 ± 37.5	0.961
HOMA-IR	4.8 ± 6.6	4.7 ± 7.0	0.944
LDL cholesterol (mg/dL)	129 ± 33	121 ± 36	0.366
HDL cholesterol (mg/dL)	50.5 ± 11.9	61.1 ± 12.4	0.001
Triglyceride (mg/dL)	235 ± 224	151 ± 128	0.200
HbA1c (%)	5.7 ± 0.9	5.8 ± 1.0	0.605
CRP (μg/dL)	254 ± 575	156 ± 226	0.323
Smoking, n (%)	14 (23.3%)	11 (29.7%)	0.484
Hypertension, n (%)	37 (61.7%)	20 (54.1%)	0.623
Diabetes/IFG, n (%)	21 (35.0%)	14 (37.8%)	0.658
Dyslipidemia, n (%)	38 (63.3%)	18 (48.6%)	0.236
Coronary artery disease, n (%)	13 (21.7%)	2 (5.4%)	0.027
Hyper uric acidemia	14 (23.3%)	10 (27.0%)	0.596
Fatty liver disease	10 (16.7%)	11 (29.7%)	0.102
Chronic kidney disease	5 (8.3%)	2 (5.4%)	0.627
OSAS, n (%)	4 (6.7%)	1 (2.7%)	0.416
Statin use, n (%)	27 (45.0%)	7 (18.9%)	0.016
Diuretic use, n (%)	12 (20.0%)	6 (16.2%)	0.658
β-blockers, n (%)	21 (35.0%)	13 (35.1%)	0.899
RAAS inhibitors, n (%)	10 (16.7%)	6 (16.2%)	0.985
Ca-channel blockers, n (%)	31 (51.7%)	14 (37.8%)	0.326
Aspirin, n (%)	21 (35.0%)	7 (18.9%)	0.117
Metabolic score (0, 1, 2, 3, and 4)	3.3 ± 0.5	1.5 ± 0.7	<0.001

Continuous variables are presented as mean ± SD whereas categorical variables are presented as counts and percentages. BMI, body mass index; HOMA-IR, homeostatis model assessment; LDL, low density lipoprotein; HDL, high density lipoprotein; HbA1c, glycated haemoglobin; IFG, impaired fasting glucose; OSAS, obstructive sleep apnea syndrome; RAAS, rennin-angiotensin-aldosterone system; Ca-channel, calcium channel.

nificantly greater in patients with MetS than those in pre-MetS (P = 0.027). However, circulating levels of BNP, a useful biomarker for heart failure, did not differ between the 2 groups.

Circulating levels of GLP-1 are increased in MetS

As shown in Figure 1, the serum GLP-1 concentration was on average 28% higher in MetS patients (7.7 ± 1.9 ng/mL)compared to pre-MetS subjects (6.0 ± 1.6 ng/mL, P < 0.001). This difference remained significant after controlling for age and sex (P = 0.010). When the data from the patients with MetS and pre-MetS were combined, GLP-1 levels were similar in men (7.0 ± 2.0 ng/mL) and women (7.2 ± 2.0 ng/mL) (P = 0.709). We next compared patient groups on the basis of the number of MetS criteria that were concomitantly present (Figure 2). Serum GLP-1 levels elevated with increasing number of MetS components (P < 0.001; Figure 2A). Among MetS components, the presence of dyslipidemia (high triglyceride and/or low HDL cholesterol) was significantly related to circulating levels of GLP-1. On the other hand, there was no correlation between the accumulation of MetS components and serum levels of GIP (Figure 2B).

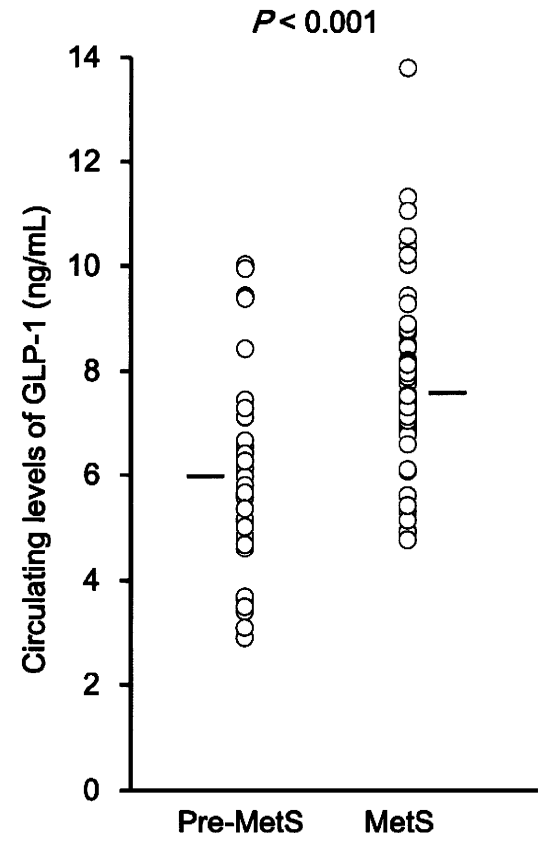

Figure 1 Circulating levels of glucagon-like peptide-1 (GLP-1) in patients with metabolic syndrome (MetS) and pre-MetS conditions. Scatter plot showing the GLP-1 (ng/mL) in patients with pre-MetS conditions (n = 37) and MetS (n = 60). The bars indicate the mean values.

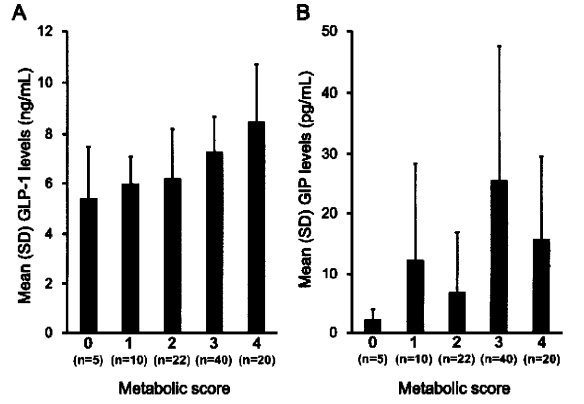

Figure 2 Association between serum levels of incretins, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), and metabolic syndrome components in patients with atherosclerosis-prone conditions. Circulating levels of GLP-1 (A) and GIP (B) were measured in high-risk patients with atherosclerosis. Metabolic score is the number of metabolic syndrome components; abdominal obesity, hypertension, dyslipidemia (high triglyceride and/or low high density lipoprotein cholesterol), and hyperglicemia (impaired glucose tolerance or diabetes). Progressive increase in GLP-1 level as a function of the number of MetS components (P < 0.001).

Association of serum GLP-1 and GIP with clinical variables

Circulating GLP-1 positively correlated with fasting serum triglycerides, uric acid, and CRP, and inversely correlated with HDL cholesterol (Table 2). Above all, serum GLP-1 was powerfully associated with a useful biomarker for systemic inflammation, serum CRP(r = 0.550, P = 0.026). In addition, circulating levels of GIP were significantly associated with GLP-1 (r = 0.383, P < 0.001). Interestingly, serum GIP was positively correlated with serum levels of LDL cholesterol and hyper uric acidemia with or without medications. However, no significant correlation was observed between serum GIP and the stating or allopurinol usage. In addition, there was no correlation between serum GIP and MetS scores. Although each variable has a large dispersion, the serum GIP concentration was on average three times higher in MetS patients (19.0 ± 45.7 pg/mL) compared to pre-MetS subjects (6.5 ± 10.2 pg/mL) (P = 0.034).

Association of serum GLP-1 with the presence of MetS

In multivariable logistic regression analyses that adjusted for age and sex, circulating GLP-1 levels in the forth quartile were significantly associated with higher odds ratios (OR) of having MetS (Table 3). This association remained significant after additional adjustment for statin use, history of coronary artery disease, and diuretic

Table 2: Spearman correlations between GLP-1, GIP, and selected variables in patients with MetS and pre-MetS conditions (n = 97).

	GLP-1 r	*P*-value	*GIP r*	*P*-value
Age (years)	0.004	0.145	0.009	0.938
Sex	0.202	0.046	-0.063	0.603
Waist	0.078	0.464	0.274	0.310
BMI	0.089	0.747	0.486	0.056
LDL cholesterol (mg/dL)	0.204	0.093	0.357	0.003
HDL cholesterol (mg/dL)	-0.229	0.049	-0.009	0.941
Triglyceride (mg/dL)	0.243	0.023	0.041	0.730
Smoking	0.118	0.326	-0.133	0.274
CRP (μg/dL)	0.550	0.026	-0.047	0.694
Plasma glucose (mg/dL)	0.064	0.554	-0.388	0.140
Plasma insulin (IU/mL)	0.069	0.571	0.088	0.499
HOMA-IR	-0.116	0.284	0.013	0.920
Diabetes/IFG	0.049	0.652	-0.132	0.278
HbA1c	-0.303	0.212	-0.029	0.810
Hypertension	0.059	0.589	0.197	0.099
Dyslipidemia	0.031	0.773	0.011	0.925
Coronary artery disease	0.093	0.391	-0.094	0.439
Hyper uric acidemia	0.133	0.219	0.276	0.019
Serum uric acid (mg/dL)	0.245	0.025	-0.023	0.849
Fatty liver disease	-0.054	0.618	-0.115	0.340
Chronic kidney disease	0.029	0.793	-0.046	0.703
OSAS	0.178	0.099	-0.014	0.908
Metabolic score	0.470	<0.001	0.157	0.189
GIP (pg/mL)	0.383	<0.001		

GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide; *r*, Spearman correlation coefficients; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein; HOMA-IR, homeostatis model assessment; IFG, impaired fasting glucose; HbA1c, glycated haemoglobin; OSAS, obstructive sleep apnea syndrome.

use, with OR of 8.04 (P = 0.015) for the fourth GLP-1 quartile.

Discussion

The present study, to the best of our knowledge, is the first to report that circulating fasting levels of GLP-1 is associated with the accumulation in MetS components. Participants with MetS had higher mean GLP-1 levels than those without MetS and participants in the fourth quartiles for serum GLP-1 had markedly higher odds of MetS compared with those in the bottom quartile (Table 3). In addition, serum GLP-1 levels were increased in participants who had greater clustering of MetS components (Figure 2A). These associations were independent of adiposity or the presence of diabetes/IFG. Furthermore, serum GLP-1 was positively correlated with serum CRP. These data suggested that MetS patients with elevated levels of GLP -1 are high-risk patients for cardiovascular disease, independent with the presence of diabetes. Gastrointestinal hormones (such as GLP-1 or GIP) that come under incretin, are secreted by intestine in response to nutrient ingestion [16]. Originally, it has been thought that they are gastrointestinal factors involved in post-ingestion augmented secretion of insulin by β cell of the pancreas [17]. According to previous literature, GLP-1 and GIP are both secreted within minutes ingestion and facilitate the rapid disposal of ingested nutrients [18]. GLP-1 promotes satiety and sustained GLP-1 receptor activation is associated with weight loss in both preclinical and clinical studies [19]. As shown in Figure 2A, circulating levels of GLP-1 were correlated with MetS score in patients with MetS. On the other hand, there was no sig-

Table 3: Association of serum GLP-1 with the presence of MetS: logistic regression analyses.

	OR (95% CI) *P* value			
	First quartile	**Second quartile**	**Third quartile**	**Fourth quartile**
GLP-1 (ng/mL)	<5.7	5.7-7.2	7.2-8.2	>8.2
Unadjusted	1	0.68 (0.14-3.35) 0.643	3.67 (0.87-15.4) 0.076	5.16 (1.23-21.56) 0.025
Age adjusted	1	0.746 (0.15-3.86) 0.746	3.75 (0.89-15.9) 0.072	5.56 (1.30-23.9) 0.021
Fully adjusted	1	0.777 (0.13-4.56) 0.780	3.57 (0.72-17.7) 0.119	8.036 (1.50-42.9) 0.015

The fully adjusted model includes age, sex and body mass index. OR, odds ratios; CI, confidence interval; GLP-1, glucagon-like peptide-1.

nificant correlation between circulating GIP and the MetS score (Table 2). According to a previous report, circulating GIP was not elevated in diabetic patients [20]. One of the reasons why there was no correlation between circulating GIP and MetS component may be due to large dispersion of circulating GIP levels in these patients. Unfortunately, we don't have enough data to determine a role on elevated levels of circulating GIP in patients with MetS. In a previous report about GLP-1 or GIP infusion to human, GLP-1 is a physical incretin and more powerful than GIP [16]. Therefore, we focused on circulating levels of GLP-1 in the present study. Earlier reports showed that GIP receptor is expressed in cells, such as β cells of the pancreas, adipocytes, or osteoblastic cells, and it plays essential roles in reserving inghested nutrients within the body in each cell. It has been reported that the control of the GIP signal can lead to improvement of MetS [21-24]. According to a previous study using mice with an inactivated GIP receptor, the duodenal hormone GIP directly links over-nutrition to obesity [22]. In a previous animal model, double incretin receptor knockout mice ($Glp1r^{-/-}$ and $Gipr^{-/-}$) fed a high-fat diet exhibited increased energy expenditure [24]. In recent studies, GLP-1 receptors are expressed in the pancreas, brain, heart, vasculature, lung, kidney, and gastrointestinal tract [17]. These data suggest that circulating levels of GLP -1 may affect to systemic metabolism in multiple organs including cardiovascular systems as a multifunctional hormone.

Recent studies have demonstrated that GLP-1 receptor agonists have wide-ranging cardiovascular actions, such as modulation of heart rate, blood pressure, vascular tone, and myocardial contractility [25]. Importantly, is appears that these agents may also have beneficial effects in the setting of cardiovascular disease. For example, GLP -1 has been found to exert cardioprotective actions in experimental models of dilated cardiomyopathy, hypertensive heart failure, and myocardial infarction. Moreover, preliminary clinical studies also indicate that GLP-1 infusion may improve cardiac contractile function in chronic heart failure patients with and without diabetes, and in myocardial infarction patients after successful angioplasty [26,27]. The precise mechanisms underlying the beneficial effects of GLP -1 in cardiac ischemia have yet to be established [25]. However, several experimental studies indicate that they occur independently of effects on glucose metabolism and may involve activation of cyclic guanosine monophosphate/cyclic adenosine monophosphate-dependent pathways and prosurvival kinases such as PI3K, Akt, glycogen synthase kinase-3β, p70s6 kinase, ERK1/2, and p38 MAPK [28-33].

Although further basic mechanistic research together with clinical investigations and metaanalyses are required, GLP-1-related drug compounds could exert beneficial effects on not only diabetes but also the cardiovascular system in patients with MetS. Several studies have shown that the magnitude of nutrient-stimulated insulin secretion is diminished in subjects with type 2 diabetes, promoting investigation as to whether incretin secretion and/or incretin action is diminished in diabetic subjects [34]. Plasma levels of GIP appear normal to increased in subjects with type 2 diabetes, whereas meal-stimulated plasma levels of GLP-1 are modestly but significantly diminished in patients with impaired glucose tolerance and in subjects with type 2 diabetes [20,35]. Based on the results of this clinical study, significant differences were found in fasting serum levels of GLP-1 between the MetS group and the pre-MetS group. On the other hand, no significant differences in GLP-1 concentration between the diabetes/IFG group and the control (normal fasting glucose) group (data not shown). The reason of the difference may come from the diabetes/IFG group including only early stage diabetes patients untreated with antidiabetic agents but not moderate to severe diabetes patients treated with oral antidiabetic agents and/or insulin.

Although the mechanism of elevated levels of GLP-1 in MetS is largely unknown, secretion of GLP-1 mostly depends upon the specific nutrient composition of the meal, and it has been reported that a particular caloric threshold or nutrient delivery rate must be reached in order to trigger significant secretion [36]. Generally, MetS patients tend to have a binge -eating disorder and it may be one of the causes of elevated levels of GLP-1 in patients with MetS. Even though the results of the study revealed no significant differences in fasting insulin and HOMA -IR between the MetS group and the pre-MetS, the levels of fasting glucose were significantly increased in the MetS group. Thus, these results suggest that the accumulation of MetS components induces the elevation of serum GLP-1 accompanied by increased levels of CRP (Table 1). Based on these observations, it is thus possible that high levels of GLP-1 may exhibit predictive information for atherosclerosis in patients with MetS.

In the present study, we found serum GLP-1 to be independently associated with several components of MetS including adiposity (BMI) and dyslipidemia (lower HDL cholesterol and/or high triglyceride levels) (Table 2). In our speculation, the association of GLP-1 with higher triglyceride levels may be secondary to increased hepatic synthesis of triglycerides under influence of glucocorticoids, glucagon, and obesity and/or MetS -induced sympathetic nervous system activation [11].

Interestingly, fasting levels of GLP-1 were significantly lower in patients after gastric bypass surgery compared with morbidly overweight controls [37,38]. Gastric bypass causes rapid resolution of insulin resistance and improved insulin secretion, perhaps mediated in part by increased GLP-1 [39], even before major weight loss has been achieved [40]. Although our study cannot demonstrate causation, it is important to note that fasting levels of GLP-1 might be linked to improve insulin resistance in obese patients. In this respect, circulating GLP-1 may be a novel biomarker for improving insulin sensitivity in high -risk patients for cardiovascular disease.

Concerning the glucose metabolism, excess glucagon secretion, abnormally accelerated gastric emptying during hyperglycemia, obesity, and increased food intake all contribute to hyperglycemia [41]. The progressive impairment of β cell function and increased insulin demand as tissue becomes insulin resistance are core pathophysiologic defects in the development of hyperglycemia in type 2 diabetes [42,43]. One of the major mechanisms of the genesis and progression of type 2 diabetes is progressive ectopic lipid deposition (e.g., in myocytes and hepatocytes, rather than in adipocytes), which induces insulin resistance, cell lipotoxicity, and diminished cell function, leading to metabolically inadequate insulin secretion [44]. Impaired release or action of GLP-1 increases excessive insulin secretion, and may play a role in the development and/or progression of type 2 diabetes in patients with MetS [45,46]. Furthermore, postprandial GLP-1 response is positively associated with changes in neuronal activity of brain areas implicated in satiety and food intake regulation in humans [47]. In a previous report, circulating levels of GLP-1 were decreased in patients with type2 diabetes [31].

In a recent study, a GLP-1 analog was effective on obese patients compared to a previous anti-obese drug, olristat. From these data, we speculate that elevating levels of GLP-1 in patients with MetS suggest the presence of systemic hyper GLP -1 concentration-induced "GLP-1 resistance" or "GLP-1 dysfunction" in those patients like as insulin resistance or leptin resistance (Figure 3). This new concept may shed further light upon the mechanisms involved in the GLP-1-inducing effect on the systemic metabolic disorder. In this point, treatment with GLP-1 mimetic agents, including DPP-4 inhibitors and GLP-1 analogs, may be a novel therapeutic strategy for patients with not only diabetes but also MetS and atherosclerosis-prone conditions.

Conclusions

In summary, higher circulating levels of GLP-1 are associated with accumulations of MetS scores and systemic inflammation, independent of the presence of diabetes. Circulating GLP-1 may be a novel biomarker for high-risk patients with MetS, and further studies are warranted to assess its utility as a predictor of incident MetS and atherogenic conditions. Our findings may suggest a novel pathophysiological mechanism underlying over nutrition, elevated levels of circulating GLP-1, and metabolic syndrome.

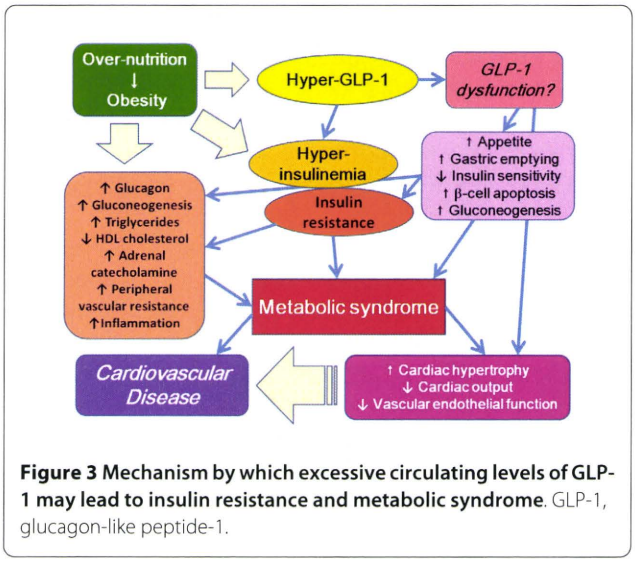

Figure 3 Mechanism by which excessive circulating levels of GLP-1 may lead to insulin resistance and metabolic syndrome. GLP-1, glucagon-like peptide-1.

Competing interests
MY- T has served as a consultant to Bayer Inc. regarding the development of drugs for the treatment of atherosclerosis. The remaining authors declare that they have no competing interests.

Authors' contributions
MY- T participated in the design of the study and performed the statistical analysis. TT conceived of the study, and participated in its design and coordination and helped to draft the manuscript. Other authors participated in enrolling patients in the study and discussion. All authors read and approved the final manuscript.

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Editorial

Vascular Protective Effects of Ezetimibe: Seeking New Therapeutic Possibilities of Ezetimibe in Vascular Disease

Atherosclerosis is known to increase the prevalence of metabolic disorders such as dyslipidemia, diabetes, and metabolic syndrome. Dyslipidemia, especially elevated levels of low-density lipoprotein (LDL)-cholesterol, is central to the management of patients at risk of atherosclerosis [1]. Traditional LDL-cholesterol lowering-therapy has focused on modifying the endogenous lipid pathway using HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, statins. Intensive LDL-cholesterol reduction with statins is associated with improved clinical outcomes in the setting of coronary artery disease [2-4], but is sometimes associated with an increased incidence of adverse drug reactions when traditional agents are used [5]. Ezetimibe (Zetia®), is a comparatively new discovered selective inhibitor of cholesterol absorption, has proved to be highly effective in lowering LDL-cholesterol with both monotherapy and combination therapy with statins [6]. Ezetimibe is expected to be the best pharmacological option for the treatment of patients unable to achieve ideal LDL-cholesterol goals with statins [7]. In recent studies, ezetimibe improved not only lipoprotein profiles but also metabolic disorder and fatty liver disease by suppressing intestinal chylomicrons production in patients with hypercholesterolemia [8, 9]. Although much evidence has revealed preventive effects of ezetimibe on atherosclerosis in several animal models, there is currently little evidence proving that treatment with ezetimibe is effective against cardiovascular events or death in humans; therefore, choosing ezetimibe to lower LDL-cholesterol is currently controversial [5].

This special issue of Current Vascular Pharmacology (CVP) is dedicated to vascular protective effects of ezetimibe focused on the recent findings of ezetimibe in vascular system. Moreover, known and proposed mechanisms of how ezetimibe may improve various vascular functions are discussed; these effects may be helpful to explain the mechanisms by which ezetimibe may protect vascular from atherosclerosis.

The purpose of this special issue of CVP is to remind readers of the evidence-based therapeutic possibility of ezetimibe and of its clinical relevance. Reviews have been written by forefront experts on data obtained from experimental animal models and/or clinical studies. A variety of excited topics are covered including ezetimibe: an update after 4 years (**Chapter 1**), effects of ezetimibe on: vascular endothelial function (**Chapter 2**), vascular inflammation from the view of immune response (**Chapter 3**), reactive oxygen species (**Chapter 4**), hepatic metabolism (**Chapter 5**), and vascular calcification and metabolism (**Chapter 6**).

We hope this special issue will help investigators in the field of prevention for cardiovascular disease and atherosclerosis to understand recent ezetimibe-related studies and find new aspects.

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Ezetimibe and Reactive Oxygen Species

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Abstract: Ezetimibe is a potent inhibitor of cholesterol absorption that has been approved for the treatment of hypercholesterolemia. Statin, 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is an inhibitor of cholesterol synthesis. Statin is the first-choice drug to reduce low-density lipoprotein (LDL)-cholesterol for patients with hypercholesterolemia, due to its strong effect to lower the circulating LDL-cholesterol levels. Because a high dose of statins causes concern about rhabdomyolysis, it is sometimes difficult to achieve the guideline-recommended levels of LDL-cholesterol in patients with high LDL-cholesterol treated with statin monotherapy. Ezetimibe has been reported to reduce LDL-cholesterol safely with both monotherapy and combination therapy with statins. Ezetimibe is especially expected to be the best pharmacological option for the treatment of patients unable to achieve LDL-cholesterol goals with statins. Reactive oxygen species (ROS) are produced at low levels to maintain physiological redox balance. Oxidative stress results when ROS production exceeds the ability of cells to detoxify ROS. Overproduction of ROS damages cellular components, including lipids, leading to decline in physiological function and cell death. Oxidative stress exacerbates atherosclerosis, the major risk factor for coronary artery disease and ischemic stroke, at every step involves the accumulation of oxidized LDL in the arteries, leading to foam cell formation, plaque development, and plaque rupture. This review focuses on the recent findings of ezetimibe-related atheroprotective effects in vasculature. Moreover, known and proposed mechanisms of how ezetimibe could improve ROS-induced pro-atherosclerotic conditions in vasculature are discussed; these effects may help to explain the mechanisms by which ezetimibe may protect vascular from atherosclerosis.

Keywords: Reactive oxygen species, atherosclerosis, dyslipidemia, inflammation, cholesterol.

1. INTRODUCTION

Atherosclerosis is the most common pathological process that leads to cardiovascular diseases, a disease of large- and medium-sized arteries that is characterized by formation of atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, inflamed endothelial cells (ECs), smooth muscle cells, leukocytes, and foam cells [1]. Although low-density lipoproteins (LDL) remain the most important and powerful risk factor for atherosclerosis, vascular inflammation- and oxidative stress-induced mechanisms of atherosclerosis have gained tremendous interest in the last two decades [1-4]. LDL are susceptible to structural modifications by oxidation, particular, particularly the small dense LDL particles [5]. Under proatherogenic conditions, nitric oxide production from ECs is reduced and the burden of reactive oxygen species (ROS) is increased [6, 7].

Ezetimibe is an epoch-making cholesterol transporter inhibitor in the small intestine to treat dyslipidemia patients with high levels of LDL-cholesterol [8]. The mechanism is absorption of both food-derived cholesterol (400-500 mg/day) and bile acid-derived reabsorbed cholesterol (800-2000 mg/day). Generally, it is difficult to achieve the target LDL-cholesterol levels by dietary therapy alone in patients with high LDL-cholesterol. HMG-CoA reductase inhibitor, statin, is an inhibitor of cholesterol synthesis. Statin is the first-choice drug to reduce LDL-cholesterol for patients with high LDL-cholesterol, because of its strong effect to lower the circulating LDL-cholesterol levels [9]. Because a high dose of statins cause concern about rhabdomyolysis, it is sometimes difficult to achieve the guideline-recommended levels of LDL-cholesterol in patients with high LDL-cholesterol treated with statin monotherapy. Ezetimibe has been reported to reduce LDL-cholesterol safely with both monotherapy and combination therapy with statins [10]. Ezetimibe is especially expected to be effective in statin-intolerant patients with high LDL-cholesterol [11].

In previous studies, ezetimibe effectively and safety reduces blood LDL-cholesterol with both monotherapy and combination therapy [12-14]. Unfortunately, it is still unclear whether or not the LDL-cholesterol-lowering by ezetimibe is effective to suppress cardiovascular events or death [15]. Whether LDL-cholesterol lowering through dual inhibition of reduced cholesterol absorption and synthesis translates to enhanced clinical benefit for reducing coronary heart disease events awaits further assessment in longer-term, outcome-based clinical trials [16-18]. The results of these coronary heart disease outcome studies will help to better clarify the clinical importance of the metabolic effects of ezetimibe with respect to LDL-cholesterol lowering and beyond.

On the other hand, recent reports have shown that the ezetimibe therapy improves hypercholesterolemia-related metabolic disorders, such as postprandial hyperlipidemia in patients with hypercholesterolemia and non-alcoholic fatty

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liver disease (NAFLD) in high-fat fed mice [19, 20]. These data suggest that ezetimibe could have multifunctional atheroprotective effects in high-risk patients with hyperlipidemia and metabolic disorder, and could be the first-choice drug to improve entire coronary risk factors accumulated in patients with metabolic syndrome. Increasing oxidative stress in accumulated fat is an early instigator of metabolic syndrome and the redox state in adipose tissue is a potentially useful therapeutic target for obesity-associated metabolic syndrome [21]. Collectively, we hypothesized that the ezetimibe therapy improves excess ROS production-caused atherosclerosis in patients with hypercholesterolemia.

In this review, we propose a new therapeutic possibility of ezetimibe as a vascular protective drug fighting against oxidative stress resulting in atherosclerosis.

2. REACTIVE OXYGEN SPECIES IN VASCULAR SYSTEM

Oxidative stress plays an important role in the pathogenesis of cardiovascular disease, cancer, renal disease, and neurodegeneration [22]. ROS are a family of molecules including oxygen, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and hypochlorous acid (HOCl). ROS contribute to vascular homeostasis at low levels as signaling molecules to mediate cell growth, migration, differentiation, and gene expression [23, 24]. In contrast, ROS at high levels induce cell death, apoptosis, and senescence. Therefore, excessive ROS production is commonly referred to as oxidative stress [25]. A large number of reports in the literature has linked oxidative stress with hypertension and atherosclerosis [26]. Vascular endothelial ROS released from a number of sources including the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, myeloperoxidase, lipoxygenases, nitric oxide synthases (NOS), and the dysfunctional mitochondrial respiratory chain may play critical roles in ROS generation [4, 27, 28]. A multi-subunit NADPH oxidase is one of the major sources of ROS in vasculature and has been linked to hypertension and to pathological states associated with uncontrolled growth and inflammation, such as atherosclerosis [29]. In vascular ECs, NADPH oxidase consists of two transmembrane proteins (p22phox and gp91phox, also called NOX2, which together form the cytochrome b_{558}) and four cytosolic proteins (p47phox, p67phox, p40phox and a GTPase Rac1), which assemble at membrane sites upon cell activation [23, 30, 31] as shown in Fig. (**1**). NADPH oxidase is activated by numerous stimuli including growth factors such as vascular endothelial growth factor (VEGF), angiopoietin-1, cytokines, shear stress, hypoxia and G-protein coupled receptor agonists including angiotensin Ⅱ (Ang Ⅱ) in ECs [29, 32, 33]. NADPH oxidase activates redox-sensitive genes (e.g. matrix metalloproteinase [MMP]; intercellular adhesion molecule [ICAM-1]; monocyte chemoattractant protein [MCP-1]; VEGF), which synthetically regulate in cell apoptosis, cell survival, inflammation, cell proliferation, hyperplasia, migration, and cell adhesion in vascular cells [28, 34]. NADPH oxidase-deficient apolipoprotein E (ApoE) knockout (ApoE (-/-)/p47phox (-/-)) mice had significantly less atherosclerosis compared with ApoE knockout mice [35]. Strong expression of NADPH oxidase subunit, Nox family proteins, is associated with increased ROS production and severity of atherosclerosis in human atherosclerosis [36]. ROS production from monocytes and macrophages, plate-

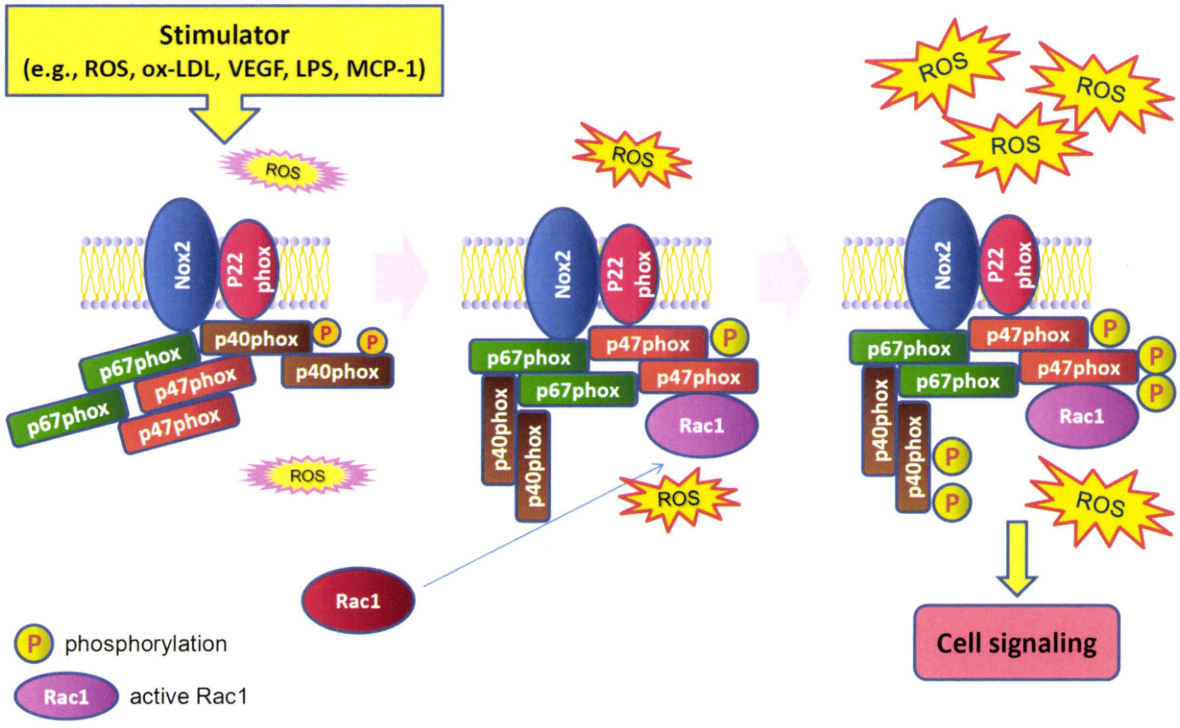

Fig. (1). Schematic diagram of the proposed concept of ROS generation in vascular endothelial cells (ECs). Nox2 (gp91phox) and its homologues (Nox1, Nox 4, and Nox5) and cytosolic components p57phox, p67phox, p40phox, and small GTPase Rac1 have been identified in ECs. ROS derived from endothelial NADPH oxidase function as signaling molecules.

lets, and vascular wall cells (e.g. ECs, smooth muscle cells) play a critical role in atherogenesis [37, 38]. During ROS-induced atherosclerosis, the activation of mitogenic signaling pathways in smooth muscle cells is also very important [37]. Antioxidants (e.g. superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) could neutralize a certain amount of ROS, but excessive ROS productions surpass the scavenging capacity of cellular antioxidant systems. Therefore, the resulting oxidative stress leads to irreversible chemical changes in lipids (peroxidation), proteins and DNA (oxidation), consequently affecting cellular dysfunction and cytotoxicity [4]. As one of the endogenous atheroprotective mechanisms, a mechanosensitive group of antioxidant enzymes, the peroxiredoxin family, is upregulated by laminar shear stress in ECs [39].

Previous studies have shown an elevated extracellular release of ROS by mononuclear cells from hypertriglyceridemic and combined hyperlipidemic patients [40, 41]. Regarding the recruitment of circulating monocytes into the vascular intima with their subsequent transformation in foam cells, foam cell formation results from monocyte-derived macrophage scavenging of modified lipoproteins that have undergone oxidative modification in the vascular wall [42, 43]. The scavenger receptor and CD36 mediated oxidized LDL uptake by macrophages lead to the initial formation of the well-defined fatty streaks in the arterial intima. Such lesions then progress to more complex ones and are prone to rupture precipitating clinical events such as ischemic heart attack and stroke [43]. ROS production by peripheral blood monocytes are upregulated in patients with hyperlipidemia [8], which may contribute to systemic oxidative stress and atherosclerosis.

3. LIPID OXIDATION AND MOLECULAR SIGNALING

Altered cellular function resulting from protein, lipid, and DNA oxidation has been implicated in vascular inflammation. Remarkably, a large number of signaling pathways, including the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase b (Akt), protein kinase C, and protein phosphatases are activated by ROS. In addition, ROS, such as peroxynitrite, hydrogen peroxide, and lipid peroxides, activate DNA binding factors (e.g. activator protein-1 [AP-1], nuclear factor-kappaB [NF-*k*B]) that, in turn, lead to transcription of several proinflammatory genes, including vascular cell adhesion molecule (VCAM)-1, ICAM-1, MCP-1, and E-selectin [44]. ROS also have been shown to activate MMPs and angiogenesis hallmarks of unstable atherosclerotic lesions [45]. Thus, ROS have been implicated in virtually every step in the atherosclerotic process [46]. Lipid oxidation is a particularly important aspect of the biochemistry of ROS in vascular system [47].

Oxysterols include oxygenated derivatives of cholesterol and plant sterols. Due to processing, heating or prolonged storage of animal-derived or deep-fried foods, the typical Western diet contains substantial quantities of oxidized cholesterol [48, 49]. Oxysterols are absorbed by the small intestine and incorporated into chylomicrons and LDL-cholesterol. Identified in the arterial wall, oxysterols may contribute to increased levels of oxidized lipoproteins in the intima [49].

LDL are susceptible to structural modifications by oxidation, particular, particularly the small dense LDL particles. The formation of lipid peroxidation derivates, such as thiobarbituric reactive substances, conjugated dienes, lipid hydroperoxides, and aldehydes, is associated with changes in apolipoprotein conformation and affects the functional properties of LDLs. The oxidized LDL (ox-LDL) is one of the most potent proatherosclerotic mediators compared to the native unmodified LDL [50, 51].

Ox-LDL receptors have been identified in the monocyte/macrophages, smooth muscle cells, and ECs. Among of all the receptors, the lectin-like receptor for ox-LDL (LOX-1) is predominantly expressed in ECs, and to a small extent in monocyte/macrophages, smooth muscle cells, and platelets. Use of a LOX-1-blocking antibody reduced almost all of the effects of ox-LDL on the EC biology described [52].

With regard to the immune mechanisms associated with the atherosclerotic process, it has been reported that ApoE knockout mice fed a standard chow diet synthesize exclusively T helper (Th)1-dependent immunoglobulin (IgG)2a anti-malondialdehyde (MDA)-modified LDL (anti-MDA-LDL), whereas antibody production is changed to Th2-dependent IgG-anti-MDA-LDL in severely hypercholesterolemic mice on a high-fat diet [53]. Therefore, dietary cholesterol administration induces a switch from Th1- to Th2-dependent autoimmune response. This could potentially be due to either a direct effect of the hypercholesterolemic state on the immune system or to a metabolic change caused by the high-fat diet, such as its content of cholic acid or different fatty acid composition [54]. To clarify the role of ROS-induced atherosclerosis, further studies including the immune response associated with hypercholesterolemia and atherosclerosis are warranted.

4. OXIDIZED LDL AS AN ATHEROGENIC CRIMINAL

Ox-LDL formation in subendothelial space of the arterial wall is a key initiating step in atherosclerosis, because it contributes foam cell generation, endothelial dysfunction, alteration of nitric oxide signaling, initiation of adhesion molecules expression in ECs that accelerate leukocyte homing to the site of atherosclerosis, and chronic vascular inflammation [55, 56]. One of the key observations that crystallized the important role of ox-LDL in atherosclerosis came from a study that showed heparin sulfate-dependent binding of ox-LDL to a subendothelial matrix [57]. Autoantibodies to ox-LDL have been found within normal/non-atherosclerosis and atherosclerosis-prone subjects. IgG autoantibodies to ox-LDL are associated with proatherogenic properties, and IgM autoantibodies to ox-LDL, including natural antibodies, have been proposed as atheroprotective properties [58]. Extensive atherosclerosis in ApoE knockout mice is associated with increased natural antibody titers to ox-LDL [58]. Most impressively, ox-LDL plays an integral role in the initiation of atherosclerosis (accumulation of inflammatory cells), to its progression (smooth muscle cell growth and proliferation), and the end event (plaque rupture and activation of platelets resulting in formation of a thrombus) [46]. In the past ten

years, immunoassays were developed using monoclonal antibodies against oxidation-dependent epitopes of LDL which made it possible to direct measurement of circulating ox-LDL. In a clinical study, 178 patients with coronary artery disease had a higher level of ox-LDL than 126 age-matched controls [59]. This finding has been corroborated in a large study comprising 1183 patients at high risk for coronary artery disease [60]. The odds ratio for high risk for coronary artery disease risk in the highest quartile of ox-LDL was 2.79, compared with the lower quintile and after adjusting for age, sex, race, LDL-cholesterol, smoking status, and C reactive protein (CRP) [60]. The Asklepios Study, investigating 2524 healthy middle-aged subjects, showed that circulating ox-LDL is affected by many biological and lifestyle factors, as well as generalized subclinical atherosclerosis [61]. Although it is still unclear whether ox-LDL elevations are a cause or a result of prevalent atherosclerosis [46], ox-LDL may be one of the useful biomarkers as a risk factor for the subsequent development of atherosclerosis in otherwise healthy subjects [62-66]. Specifically, circulating the ox-LDL level reflects early atherosclerotic changes and metabolic disorders including diabetes and obesity [67]. *In vitro* exposure to ox-LDL increased NF-*k*B activity in mononuclear cells, suggesting a pathogenic role of circulating ox-LDL in the exacerbation of oxidative stress [68]. The oxidative modification of LDL cholesterol to ox-LDL is thought to be a key initiating step in the development of atherosclerosis. Ox-LDL decreases the gene expression of the EC NO synthase (eNOS) and enhances ROS generation in ECs [69]. Moreover, ox-LDL also activates inflammatory cells and facilitates release of a large number of growth factors from monocytes and macrophages [70]. Later, ox-LDL acts as a persistent proinflammatory trigger for the progression of atherosclerosis and plaque rupture (atherothrombosis), responsible for the downstream clinical sequelae. Interestingly, ox-LDL is also a risk marker for early ventricular remodeling, independent of classic risk factors, lifestyle, inflammation, and prevalent vascular damage [61]. One mechanistic explanation for the observed effects on cardiac structure and function can be related to ox-LDL directly-induced myocardial damage [71] and intense contractile and electrophysiological changes [72]. Although the causality remains unproven, the inhibition of ox-LDL may be a useful therapeutic strategy to prevent non-ischemic cardiac failure in patients with atherosclerosis-prone.

5. EFFECTS OF EZETIMIBE IN LOW-DENSITY LIPOPROTEIN-CHOLESTEROL LOWERING AND BEYOND

Clinically, the ezetimibe therapy diminished circulating levels of total cholesterol and LDL-cholesterol in patients with primary hypercholesterolemia [73-75]. In monotherapy studies, ezetimibe generally decreased triglyceride (TG) levels compared with placebo [73, 76, 77]. Moreover, ezetimibe monotherapy has been reported to significantly decrease fasting levels of TG, apolipoproteinB (apoB)-48, and remnant lipoprotein cholesterol in patients with type IIb hypercholesterolemia [19]. When co-administered with statins, ezetimibe resulted in greater additional reductions in TGs levels compared with placebo [78-85]. The mechanism of ezetimibe-induced reduction of TG levels is still unclear. One of the mechanisms that ezetimibe improves lipoprotein profiles may be the suppression of intestinal chylomicrons (CM) production. Circulating TG are mainly found in TG-rich lipoproteins (TRL) consisting of CM and very low-density lipoproteins (VLDL). CM assemble dietary cholesterol, TG, and apoB-48 in enterocytes; and VLDL assemble endogenous hepatic TG, cholesterol, and apoB-100 in hepatocytes. In the study, the administration of ezetimibe improved endogenous and exogenous TRL profiles by suppressing postprandial intestinal production of CM and reducing the fasting hepatic cholesterol pool [19]. Thus, ezetimibe treatment could be a favorable therapeutic option for patients with elevated VLDL, LDL, and remnant lipoproteins, as in metabolic syndrome patients with hypercholesterolemia. Indeed, several studies have shown that ezetimibe improved lipid metabolism in obese patients with dyslipidemia and in animal models for metabolic syndrome [86-88]. In our clinical data, ezetimibe dramatically improved lipid profiles and a biomarker of oxidative stress in high-risk patients with hypercholesterolemia (Table **1**). The study was undertaken to investigate the effect of ezetimibe (10 mg/day) in combination with a statin on hypercholesterolemia in 12 high-risk patients of coronary artery disease. Ezetimibe add-on therapy induced a mean reduction in LDL-cholesterol of 27% ($p = 0.004$) at the 22nd week.

6. ATHEROPROTECTIVE MECHANISMS OF HIGH-DENSITY LIPOPROTEIN AND EZETIMIBE

Cholesterol efflux from peripheral tissues into plasma, then to the liver, in which cholesterol and its metabolic products (e.g. bile acids) are excreted into bile, is termed reverse cholesterol transport [89]. HDL-cholesterol mediates most reverse cholesterol transport and thus influences the amount of cellular components under normal and pathogenic conditions. HDL also has antioxidant, anti-inflammatory, anti-apoptotic, and vasodilatory properties. For instance, HDL inhibits expression of adhesion molecules and reduces leukocyte homing to arterial endothelium [90, 91]. Infusion of reconstituted HDL, suppression of cytokine, and chemokine production was observed in an inflammatory porcine model [92]. The mechanism of which may include that HDL-associated lysosphingolipids inhibit NADPH oxidase-dependent ROS generation and MCP-1 production in a process that requires coordinate signaling through sphingosine 1-phosphate (S1P)3 and scavenger receptor type B1(SR-B1) receptors [93].

The level of plasma HDL-cholesterol is inversely associated with the risk of developing atherosclerosis [94, 95], apparently in part because of its role in promising reverse cholesterol transport [96-99]. Study participants from the Framingham Study at the 80th percentile of HDL cholesterol were found to have half the risk of coronary heart disease developing when compared with subjects at the 20th percentile of HDL cholesterol [100]. One of the mechanisms of HDL-induced atheroprotective effect is the regulation of ROS-related vascular inflammations. HDL-associated lysosphingolipids inhibit NADPH oxidase-dependent MCP-1 [93]. Amazingly, HDL-cholesterol could lose their usual atheroprotective properties through specific chemical and structural alterations and could play a proinflammatory role by alteration of reverse cholesterol transport, enhanced oxi-

Table 1. Effect of Ezetimibe on Circulating Biomarkers in Patients with Hypercholesterolemia

Biomarkers	Ezetimibe (-)	Ezetimibe (+)	*P*-Value
Triglyceride (mg/dL)	205 ± 118	173 ± 66	0.355
LDL-cholesterol (mg/dL)	139 ± 16	102 ± 14	0.004
HDL-cholesterol (mg/dL)	55 ±7	56 ± 11	0.610
FFA	412 ± 250	378 ± 191	0.706
RLP-cholesterol (mg/dL)	11.2 ± 7.4	8.8 ± 5.4	0.187
Apolipoprotein B (mg/dL)	107 ± 16	90 ± 10	0.074
Apolipoprotein AI (mg/dL)	142 ± 12	147 ± 19	0.455
MDA-LDL (mg/dL)	112 ± 15	92 ± 29	0.222
Glucose (mg/dL)	123 ± 26	111 ±17	0.153
HbA1c (%)	6.1 ± 0.7	6.0 ± 0.8	0.529
hs-CRP (mg/dL)	0.10 ± 0.06	0.16 ± 0.20	0.407
dROMs (U Carr)	364 ± 46	335 ± 50	0.044

Data were shown as mean ± SD. The Student's paired *t*-test was used for pairwise comparisons between values before and after administration of ezetimibe for 22 weeeks (n = 12). LDL, low density lipoprotein; HDL, high density lipoprotein; FFA, free fatty acid; RLP, remnant lipoprotein; MDA, malondialdehyde-modified; HbA1c, haemoglobinA1c; hs-CRP, high sensitivity C-reactive protein; dROMs, derivatives of the reactive oxidative metabolites.

dation of LDL-cholesterol, and increased vascular inflammation linked to atherosclerosis [101].

In ApoE knockout mice, ezetimibe administration (5 mg/kg/day) for 3 months markedly reduced plasma levels of LDL-cholesterol (78% reduction) and increased HDL-cholesterol (87% increase) in high-fat diet mice [102]. Ezetimibe monotherapy elicits modest increases in HDL-cholesterol levels. In several ezetimibe monotherapy trials, 5 and 10 mg/day of ezetimibe provided additional increases in HDL-cholesterol from baseline levels compared with placebo (2.2% to 3.5%; $p < 0.05$ to $p < 0.01$) [73, 76, 77, 103]. The modest increase in HDL-cholesterol levels induced by ezetimibe may be attributed to the liver, which subsequently enhances the clearance of TRL. A reduction in the levels of TRL, which are acceptors of cholesterol transferred from HDL particles by cholesteryl ester transfer protein (CETP), may contribute to a reduced rate of cholesterol transfer, thereby increasing levels of HDL-cholesterol [104, 105]. In our clinical data, regarding patients with hyper LDL-cholesterolemia without low HDL-cholesterol, ezetimibe treatment had a tendency to increase serum HDL-cholesterol but not significantly (Table 1).

To determine atheroprotective effects of ezetimibe, we evaluated ratios of proatherogemic to antiatherogenic lipoprotein measurements, LDL to HDL cholesterol (LDL/HDL cholesterol) and apolipoprotein B to apolipoprotein A-I (apolipoprotein B/A-I) [106]. As shown in Fig. (**2**), ezetimibe improved the ratio of LDL/HDL cholesterol and apolipoprotein B/A-I. Most importantly, the most impressive data is that ezetimibe improved the ratio of LDL/HDL cholesterol (by 41%; from 2.7 to 1.6; $p < 0.001$) as shown in Fig. (**2A**). According to a recent report in the literature, the lipid ratio of LDL/HDL is better monitoring predictors than single standard lipids including total cholesterol, LDL-cholesterol, and HDL-cholesterol [107]. Regarding initial risk measurements, several previous cohort studies and a meta-analysis study suggest that the ratios of LDL/HDL also have greater independent predictive values for coronary heart disease than individual serum total cholesterol or LDL-cholesterol levels [108-112]. In the current therapy for hypercholesterolemia, ezetimibe is the most powerful agent to improve the LDL/HDL ratio compared to any statin monotherapy.

Furthermore, ezetimibe add-on therapy significantly reduced the ratio of apolipoprotein B/A-I in high-risk patients with hypercholesterolemia as shown in Fig. (**2B**). The add-on therapy with ezetimibe "significantly" reduced the apolipoprotein B/A-I ratio in high-risk patients with hypercholesterolemia (by 25%; from 0.8 to 0.6; $p = 0.029$). Patients who were treated adequately according to current guidelines (i.e. LDL cholesterol $\leq$ 100 mg/dl) still had residual major car-

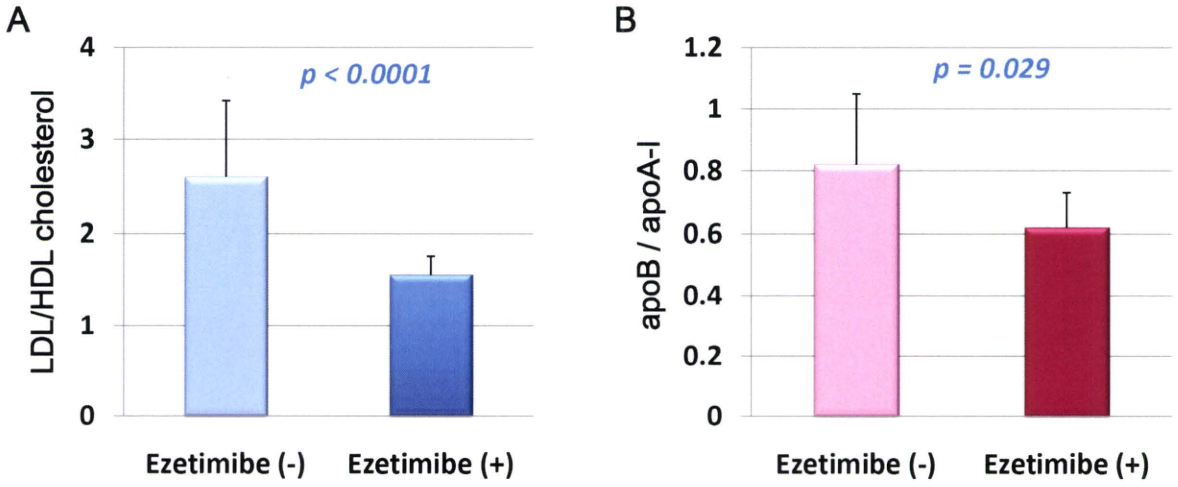

Fig. (2). Ezetimibe improved lipid profiles in patients with hypercholesterolemia. A, Ezetimibe improved LDL/HDL- cholesterol ratio in patients with hypercholesterolemia. B, Ezetimibe improved apolipoproteinB (apoB) to apolipoproteinA-I (apoA-I) ratio in patients with hypercholesterolemia. LDL-cholesterol, HDL-cholesterol, apoB, and apoA-1 were measured before and after 22-week ezetimibe administration in patients with athrosclerosis-prone hypercholesterolemia (n = 12; mean age, 63 ± 3 years; 50% women). Bars represent the mean value of the ratio ±SD.

diovascular event risks that could be recognized by the evaluation of levels of non-HDL cholesterol (the sum of the cholesterol concentration in all proatherogenic lipoproteins [VLDL, IDL, and LDL particles]) or apolipoprotein B (the major apolipoprotein of these particles) [110]. On-treatment levels of non-HDL cholesterol and apolipoprotein B are considered to be more closely associated with cardiovascular outcome than levels of LDL cholesterol [113]. These data suggest that ezetimibe not only reduce serum levels of LDL cholesterol but also improve total lipid profiles.

7. MOLECULAR TARGET OF EZETIMIBE AS AN ATHEROPROTECTIVE DRUG

Ezetimibe, a novel lipid-lowering agent, selectively inhibits intestinal cholesterol absorption, reducing total cholesterol and triglyceride levels and also reducing the development of atherosclerosis in ApoE knockout mice [114, 115]. According to data from apoE and eNOS double knockout mice, lipid lowering with ezetimibe potentially reduced atherosclerosis and vascular inflammation independent of eNOS [116]. A sterol transporter, Niemann-Pick C1-Like 1 (NPC1L1) is involved in subcellular cholesterol trafficking and plays a critical role in the absorption of intestinal cholesterol [117, 118]. NPC1L1-deficient mice exhibit a substantial reduction in absorbed cholesterol, on which ezetimibe had no effect [118]. Thereafter, the molecular target of ezetimibe was revealed to be NPC1L1, which is a critical mediator of cholesterol absorption and an essential component of ezetimibe-sensitive pathway [115]. Ezetimibe was reported to bind specifically to a single site in mammalian enterocyte brush border membranes and to human embryonic kidney cells expressing NPC1L1 [115]. In an individual with dyslipidemia who did not respond to treatment with ezetimibe, compound heterozygosity for 2 rare polymorphisms of the NPC1L1 gene was identified [119].

NPC1L1 is widely expressed in many human tissues, with the highest expression in the small intestine as well as in the liver [120, 121]. Therefore, ezetimibe inhibits cholesterol absorption in the small intestine, reduces enterohepatic circulation of cholesterol, reduces uptake of free cholesterol in hepatocytes, and should affect metabolic pathways in the liver [122]. Ezetimibe improved hepatic insulin signaling as well as hepatic steatosis in Zucker Obese fatty (ZOF) rats. Ezetimibe also restored insulin sensitivity in steatotic hepatocytes *in vitro* by a reduction in hepatic ROS generation, Janus-family tyrosine kinase (JNK) activation, and endoplasmic reticulum stress. In addition, ezetimibe recovered insulin-induced Akt activation, and reduced gluconeogenic genesin the liver of ZOF rats and cultured steatotic hepatocytes [122]. Other studies have shown that ROS-dependent activation of JNK plays a role in the developing insulin resistance [123-125]. In recent clinical studies, ezetimibe treatment has been considered as an effective therapeutic option for NAFLD [120, 126, 127]. Several reports have concluded that ezetimibe monotherapy not only protects against high fat diet-induced dyslipidemia but also attenuates liver steatosis in an experimental NAFLD model [88, 128]. In ApoE knockout mice, liver weight was significantly decreased and lipid accumulation in the liver was also dramatically inhibited in the ezetimibe-treated group [102]. These accumulating data suggest that the inhibition of NPC1L1-dependent cholesterol uptake by ezetimibe may be a suitable therapeutic target for treatment of not only hypercholesterolemia but also broader aspects of metabolic disorders in patients with type 2 diabetes and/or metabolic syndrome as shown in Fig. (**3**).

8. BIOMARKERS FOR OXIDATIVE STRESS AND EZETIMIBE

With regard to ezetimibe, several clinical studies have already revealed that ezetimibe therapy improves biomarkers for oxidative stress [129, 130]. Furthermore, ezetimibe decreased serum asymmetric dimethylarginine (ADMA) levels, urinary excretion levels of 8-hydroxydeoxyguanosine (8-OHdG), markers of oxidative stress, and proteinuria in pa-

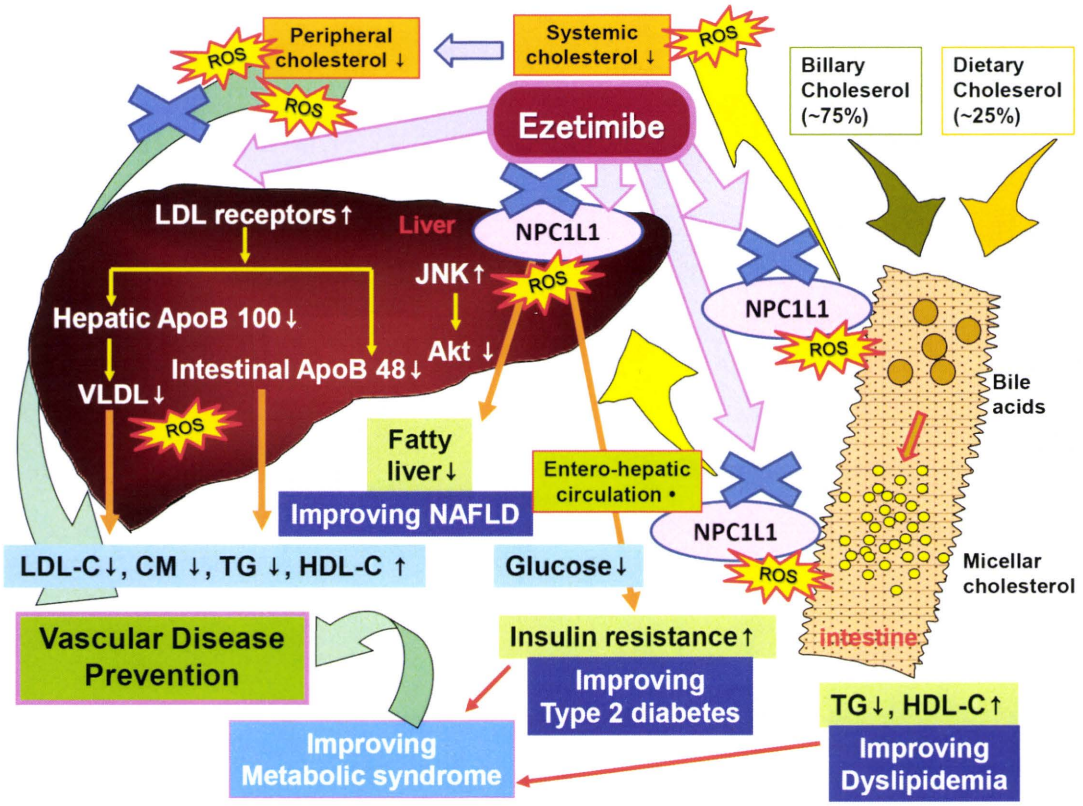

Fig. (3). Schematic diagram of the proposed mechanisms of ezetimibe-induced atheroprotective effects through reactive oxygen species (ROS)-related pathways. LDL-C: low-density lipoprotein-cholesterol; ox-LDL: oxidized LDL; VEGF: vascular endothelial growth factor; LPS: lipopolysaccharide; MCP-1: monocyte chemotactic protein-1; CM: chylomicrons; TG: triglyceride; HDL-C: high-density lipoprotein-cholesterol; NAFLD: non-alcoholic fatty liver disease; NPC1L1: Nieman-Pick C1-like 1; apoB: apolipoproteinB; VLDL: very low-density lipoprotein; JNK: Janus-family tyrosine kinase.

tients with non-diabetic chronic kidney disease with dyslipidemia [130].

Based on the site of ROS release and their activity, a large number of biomarkers of oxidative stress have been proposed, such as ox-LDL, antibodies to ox-LDL, F2-isoprostanes, thiobarbituric acid reactive substances (TBARS), free oxygen radical monitor (FORM) (derivatives of reactive oxidative metabolites, dROMs), 8-OHdG [46, 131-135].

Of all others, the FORM assay detecting organic peroxides known as the dROMs test was available [136, 137]. This assay is relatively inexpensive, simple, easy, and can be performed in 5 minutes. In previous reports, the dROMs assay was used to assess the effectiveness of various antioxidant treatment strategies [137, 138]. To examine whether ezetimibe improves oxidative stress or not, we studied ezetimibe add-on therapy with a statin. The ezetimibe add-on therapy remarkably reduced dROMs levels in high-risk patients with hypercholesterolemia as shown in Fig. (**4**). Considering that ezetimibe monotherapy or a combination therapy with simvastatin decreased LDL tendency to peroxidation, ezetimibe may have favorable pleiotropic effects beyond the LDL-cholesterol lowering [129, 130]. These data suggest that ezetimibe could contribute to atheroprotective properties through effective anti-oxidant actions. Since ox-LDL induces expression of a NADPH oxidase, Nox2, and ROS generation in vascular ECs [139], ezetimibe may attenuate ROS production through reduction of circulating ox-LDL in patients with hypercholesterolemia.

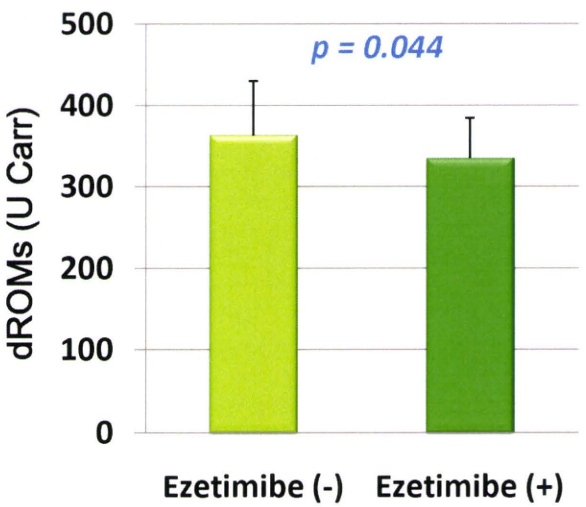

Fig. (4). Ezetimibe reduced a circulating oxidative marker, derivatives of reactive oxidative metabolites (dROMs), in patients with atherosclerosis-prone with hypercholesterolemia. Measurements of dROMs were assessed before and after 22-week ezetimibe administration in patients with athrosclerosis-prone hypercholesterolemia (n = 12; mean age, 63 ± 3 years; 50% women). Bars represent the mean value of the ratio ±SD.

9. EFFECTS OF EZETIMIBE TO IMPROVE ENDOTHELIAL FUNCTION IN PATIENTS WITH DYSLIPIDEMIA

Endothelial dysfunction is the initial step of atherosclerosis. A recent report demonstrated that ezetimibe treatment attenuated vascular functions such as endothelial dysfunction, oxidative stress, and inflammation in high-fat fed ApoE-deficient mice [102]. Ezetimibe treatment markedly inhibited the development of lipid-rich plaque and also significantly improved endothelial dysfunction assessed by the vasodilator response to acetylcholine [140], accompanied by inhibition of interleukin-6 mRNA and an increase in eNOS mRNA in the aorta. Furthermore, ezetimibe suppressed ROS generation and the ubiquitination-proteasome system in the aorta.

In our clinical data, after 22 weeks of ezetimibe add-on combination therapy with a statin, reactive hyperemia index (RHI) by the EndoPAT™, a fingertip peripheral arterial tonometry device [141, 142], was improved in high-risk patients with hypercholesterolemia as shown in Fig. (**5**). According to a previous study, an RHI < 1.35 was found to have a sensitivity of 80% and a specificity of 85% to identify patients with coronary endothelial dysfunction [143]. Ezetimibe add-on therapy achieved effective RHI-improvement (mean RHI 1.32 to 1.53, p = 0.020) in high-risk patients with hypercholesterolemia.

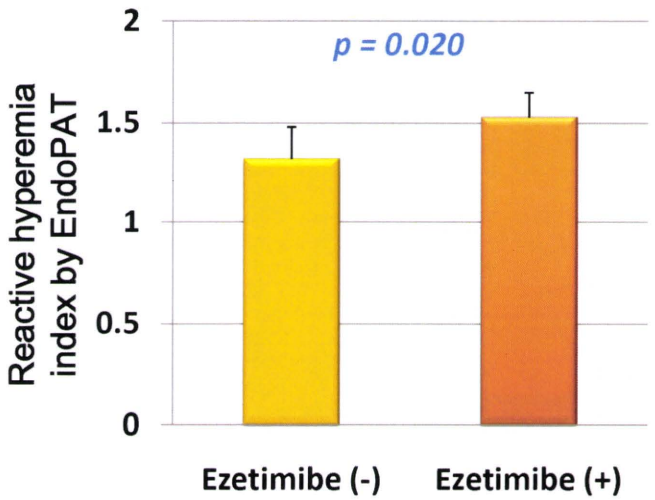

Fig. (5). Ezetimibe improved reactive hyperemia index (RHI) by EndoPAT™ in patients with hypercholesterolemia. Using a fingertip peripheral arterial tonometry device, we measured digital pulse amplitude in patients with athrosclerosis-prone hypercholesterolemia (n = 12; mean age, 63 ± 3 years; 50% women) for 5 minutes at baseline and after a reactive hyperemia induced by 5-minute forearm cuff occlusion. RHI were measured before and after 22-week ezetimibe administration in patients with hypercholesterolemia. Bars represent the mean value of the ratio ±SD.

Considering mechanisms of improving endothelial function, cholesterol lowering may be more important than pleiotropic effects of lipid-lowering drugs for improvement in endothelial function in patients with dyslipidemia and coronary artery disease [144].

Through the inhibition of chemokine (C-C motif) receptor 2 (CCR)2-mediated recruitment of monocytes into the vessel wall by activating intracellular signaling pathways, ox-LDL activates peroxisome proliferator-activated receptor gamma (PPARγ) [145]. However, endothelial PPARγ regulates vascular NO production and contribute to endothelial dysfunction [146]. Activation of PPARγ attenuated both metabolic syndrome and atherosclerosis in aging mice through a novel antioxidant mechanism (DJ-1 and forkhead box, subgroup O family, FOXOs; nuclear factor [erythroid-derived 2]-like 2, Nef2) to enhance vascular antioxidant responses in lipoprotein receptor knockout mice [147]. Ezetimibe-affected vascular endothelial function may be attributed not only to its blocking of cholesterol absorption but also to decreased ROS-mediated NO inactivation and enhanced antioxidant systems.

CONCLUSIONS

In conclusion, ezetimibe inhibits NPC1L1 controlling cholesterol absorption in the intestine, reduces excessive ROS production, and prevents atherosclerosis in vasculature. Moreover, ezetimibe improves NPC1L1 contributing hepatic insulin resistance and fatty liver through suppressing free cholesterol accumulation and subsequent ROS generation. As an atheroprotective drug, ezetimibe may be a suitable therapeutic target for treatment of not only hypercholesterolemia but also broader aspects of metabolic disorders in patients with type 2 diabetes and/or metabolic syndrome.

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LIST OF ABBREVIATIONS

8-OHdG	=	8-hydroxydeoxyguanosine
ADMA	=	Asymmetric dimethylarginine
Akt	=	Protein kinase b
Ang ‖	=	Angiotensin ‖
AP-1	=	Activator protein-1
apoA-1	=	Apolipoprotein A-1
apoB	=	ApolipoproteinB
apoE	=	Apolipoprotein E
CCR	=	Chemokine (C-C motif) receptor
CETP	=	Cholesteryl ester transfer protein
CM	=	Chylomicrons
CRP	=	C reactive protein
DJ-1	=	Parkinson disease 7 (PARK7)
dROMs	=	Derivatives of reactive oxidative metabolites
eNOS	=	Endothelial cell NO synthase
ECs	=	Endothelial cells
FORM	=	Free oxygen radical monitor