



Serum myeloperoxidase/paraoxonase 1 ratio as potential indicator of dysfunctional high-density lipoprotein and risk stratification in coronary artery disease

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ABSTRACT

Objective: Granular leukocyte-derived myeloperoxidase (MPO) promotes oxidation of lipoproteins, while paraoxonase 1 (PON1) has antioxidant properties for high-density lipoprotein (HDL). We evaluated their effects on coronary risk stratification and function of lipoproteins.

Methods and results: A total 158 patients who had previously undergone percutaneous coronary intervention and who had been hospitalized for coronary re-angiography were enrolled. Coronary lesions (restenosis or de novo lesion) were observed in 84 patients but not associated with conventional lipid profile. In contrast, serum MPO levels and PON1 activities were significantly associated with the prevalence of coronary lesions. The high MPO/PON1 ratio, when cutoff values were set at 1.59, was independently correlated with restenosis (odds ratio 6.4, 95% CI 2.2–19.3, $P = 0.001$) and de novo lesions (odds ratio 3.5, 95% CI 1.3–9.4, $P = 0.014$). We isolated HDL from patients with high or low MPO/PON1 ratio, and compared anti-inflammatory properties of HDL. Human umbilical vein endothelial cells were stimulated with inflammatory cytokine, and the expression of vascular cell adhesion molecule-1 (VCAM-1) was evaluated. HDL isolated from patients with low serum MPO/PON1 ratio inhibited VCAM-1 expression significantly greater than that with high MPO/PON1 ratio. We also demonstrated that the cholesterol efflux capacity of apolipoprotein B-depleted serum from patients with high MPO/PON1 ratio was significantly decreased than that with low MPO/PON1 ratio.

Conclusions: MPO/PON1 ratio could be a useful marker for secondary prevention of coronary artery disease through modulation of HDL function.

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1. Introduction

Although high plasma level of low-density lipoprotein cholesterol (LDL-C) is an established risk factor for coronary artery diseases (CAD), the adequate LDL-lowering therapy with statins has been shown to reduce the prevalence of CAD by 30–40% at most [1,2]. LDL displays several phenotypes such as oxidized LDL and small dense LDL, which accelerate atherosclerosis much greater than native LDL,

therefore the modulation of LDL size and subclasses may be a next therapeutic target for the residual cardiovascular risk [3]. On the other hand, high-density lipoprotein cholesterol (HDL-C) is a negative risk factor for CAD [4]. HDL exhibits a variety of anti-atherogenic functions including anti-inflammatory and anti-oxidative as well as promoting reverse cholesterol transport [5]. However, it has been reported that HDL may lose its anti-atherogenic properties and become pro-atherogenic (dysfunctional) under conditions such as inflammation, diabetes, and oxidative stress [5]. These lines of evidence suggest that the function of lipoproteins may significantly modulate and predict the progression of CAD in addition to the quantity of lipoproteins.

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Myeloperoxidase (MPO) is a heme peroxidase, which is produced from granular leukocytes. Besides its antibacterial effects, MPO causes oxidative- or chemical-modification against circulating lipoproteins [6]. In addition, MPO generates reactive oxygen and nitrogen species which facilitate lipid peroxidation, protein nitration, and protein carbamylation [6]. LDL which undergoes these modifications promotes foam cell formation. On the other hand, the modified HDL loses its cholesterol efflux activity and anti-inflammatory properties [7]. Through these functional changes, MPO may promote the progression of atherosclerosis.

Paraoxonase 1 (PON1) is one of the major HDL-associated proteins. PON1 is strongly lipophilic and co-exists with apolipoprotein (apo)A-I and apo-J in HDL particles. PON1 hydrolyzes organophosphates and its activity is stabilized in the presence of the apolipoprotein [8]. PON1 inhibits oxidation of LDL, and reduce oxidative stress in blood vessels [8]. In fact, previous studies have shown that the activities of PON1 are negatively associated with the prevalence of cardiovascular events [9]. Given the function of lipoproteins is impaired by MPO and improved by PON1, the plasma level or activity of MPO and PON1 is a marker and modulator of not only lipoprotein functions but also initiation and progression of CAD. However, it has not been fully elucidated whether these molecules, alone or in combination, predict the prevalence of CAD. In the present study, therefore, we evaluated serum MPO level and PON1 activity in patients with chronic CAD, and validated a hypothesis that the MPO/PON1 ratio could be a useful marker for secondary prevention of CAD through modulation of HDL functions.

2. Materials and methods

2.1. Study subjects

From April 2008 to March 2010, we enrolled 158 consecutive patients who had previously undergone successful percutaneous coronary intervention (PCI) with stenting one or more times and who had been admitted to Kobe University Hospital for the purpose of coronary angiography (CAG) because of 6-month follow-up or stable angina or inducible ischemia (CAD group). On the other hand, 174 patients without past history of CAD were enrolled as a non-CAD group in the same period. In addition, the CAD group was classified according to the following definition: patients who showed restenosis in the original stented segment (in-stent restenosis; ISR group); patients with occurrence of other non-target coronary atherosclerotic lesions (de novo lesion group); and patients with neither ISR nor de novo lesion (no lesion group). ISR and de novo lesions were defined as displaying luminal stenosis $\geq 75\%$ and demonstrating ischemia in the perfusion area of narrowed coronary by stress myocardial scintigram. Weight and height were measured and body mass index (BMI) was obtained by dividing a patient's weight by their height squared. Patients who had acute coronary syndrome, renal failure (creatinine >3.0 mg/dL), and a history of cancer in the previous 5 years were excluded. To exclude inflammatory diseases (e.g., infections, malignancies, autoimmune diseases), patients whose serum C-reactive protein (CRP) was more than 1.0 mg/dL were also not assigned because MPO is produced from granular leukocytes, and serum MPO levels might be influenced by inflammatory conditions. All patients gave written informed consent, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. The investigation conforms to the principles outlined in the Declaration of Helsinki.

2.2. Blood chemistry

Serum was obtained after overnight fast. LDL-C, HDL-C, apoA-I, apoA-II, apoB, apoC-II, apoC-III, apoE, glucose, hemoglobin (Hb) A1c, high sensitivity C-reactive protein (hsCRP), and triglyceride (TG) were measured using standard methods at Kobe University Hospital. PON1 paraoxonase activity was measured using paraoxon as the substrate by SRL (Kobe, Japan). The rate of generation of p-nitrophenol was determined spectrophotometrically. Residual serum was stored at -80°C for assays of MPO and PON1 arylesterase activity. Arylesterase activity was measured as follows; Serum was preincubated with 10 μM eserine (Sigma, St. Louis, MO, USA) for 10 min at room temperature for the inhibition of butyrylcholinesterase activity. After adding 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM CaCl_2 and 1 mM phenyl acetate (Wako Chemicals, Richmond, VA, USA), the rate of hydrolysis of phenyl acetate was determined spectrophotometrically at 270 nm in an automated Shimadzu UV-1600, UV-visible Spectrophotometer (Shimadzu, Kyoto, Japan) [10]. Serum MPO levels were measured by human MPO ELISA kit (Hycult Biotechnology, Uden, Netherlands) as per manufacturer's protocol.

2.3. Inhibition of tumor necrosis factor- α induced vascular cell adhesion molecule-1 expression by HDL

HDL fraction was obtained from the serum with MPO/PON1 ratio <1 or >3 by ultracentrifugation as described elsewhere [11]. Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Manassas, VA, USA). Subconfluent HUVECs were seeded in a 24 well-plate at 1.2×10^5 cells/well. After 16 h, the media was replaced with new one containing human HDL (100 μg protein/well) which was purified from serum with MPO/PON1 ratio <1 or >3 , or bovine serum albumin (control, 100 μg protein/well). HUVECs were incubated for 16 h, then stimulated with or without tumor necrosis factor (TNF)- α (10 ng/ml) for 6 h. Experiments were terminated by aspiration of the medium and washing cells twice with 500 μl of cold phosphate buffered saline. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by cDNA preparation using reverse transcript agent, then quantitative real-time polymerase chain reaction (PCR) was performed. PCR primers for human vascular cell adhesion molecule-1 (VCAM-1) and glyceraldehyde 3-phosphate dehydrogenase were purchased from Takara-Bio Perfect Real Time Support System (Takara, Shiga, Japan).

2.4. Cholesterol efflux assays

To quantify HDL efflux capacity, apolipoprotein B-depleted serum was prepared from patients with MPO/PON1 ratio >3 or <1 as previously reported [12]. THP-1 human monocyte cells were seeded into 12 well plates at density of 3×10^6 cells per well. Cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (Sigma, final concentration 100 ng/ml) for 60 h. Cellular cholesterol was labeled with 1 μCi of [^3H]-cholesterol for 12 h. Then cells were washed with PBS and incubated for 18 h with medium containing 0.2% BSA and TO-901317 (Cayman, final concentration 10 μM /l) to up-regulate ABC transporters. Cells were washed again with PBS and incubated for 4 h with medium containing 2% apolipoproteinB-depleted serum. After 4 h, medium was collected and centrifuged at 15,000 rpm for 5 min and then aliquots of supernatant were counted in liquid scintillation. The cell layer was washed twice with PBS and incubated with isopropanol for 1 h at room temperature, and radioactivity was measured as for the supernatant. Cholesterol efflux was given as the following; the proportion of [^3H]-cholesterol counts in the medium to the total

counts present on the well (i.e., sum of medium and cells) were calculated, and then background values (i.e., the efflux in medium without apolipoprotein B-depleted serum) were subtracted from the respective experimental values. All samples were analyzed in triplicate.

2.5. Statistical analysis

All statistical analyses were performed using Stata10 (College Station, TX, USA). All data are expressed as mean $\pm$ s.d., unless otherwise specified. *P* value for two groups was determined by Student's *t*-test or the Mann–Whitney test according to the data distribution, with or without normality. The χ^2 -test was used to compare categorical variables between the groups. The differences among multiple groups were analyzed by Kruskal–Wallis test. Multiple regression analyses were performed to evaluate the relationship between the prevalence of coronary lesions (ISR, de novo lesions, or both) in the CAD group and other clinical parameters. *P* value <0.05 was considered significant. The receiver operating characteristic curve (ROC curve) was used to determine MPO/PON1 ratio cut-off point. In order to investigate if MPO/PON1 ratio provide more effective CAD risk stratification than serum MPO levels alone, we calculated net reclassification improvement (NRI) and integrated discrimination improvement (IDI) according to Pencina et al. [13]. The associations of MPO and PON1 with other clinical parameters were estimated using linear regression analysis.

3. Results

3.1. Conventional risk markers do not associate with the prevalence of coronary lesions in patients with a past history of CAD

In 158 patients who underwent CAG re-examination, 44 patients showed ISR, 40 patients had de novo lesions, and 74 patients did not show either ISR or de novo lesions. There were no significant differences in the baseline characteristics, including what

Table 1
Baseline characteristics of the CAD patients.

	No lesion (<i>n</i> = 74)	De novo (<i>n</i> = 40)	ISR (<i>n</i> = 44)	<i>P</i> -value
Age (years)	67.4 $\pm$ 10.0	68.5 $\pm$ 10.9	67.1 $\pm$ 9.3	0.693
Sex (Male %)	74.2	84.9	89.1	0.059
Family history (%)	25.6	26.0	33.3	0.605
Current smoking (%)	28.6	23.1	18.5	0.384
Hypertension (%)	89.1	83.0	80.0	0.292
Diabetes mellitus (%)	44.6	57.7	57.4	0.191
Dyslipidemia (%)	80.2	76.9	79.6	0.893
Waist (cm)	88.5 $\pm$ 9.2	88.3 $\pm$ 8.9	88.7 $\pm$ 9.8	0.964
BMI (kg m ⁻²)	24.6 $\pm$ 3.1	24.4 $\pm$ 3.7	24.8 $\pm$ 4.2	0.857
SBP (mmHg)	126.2 $\pm$ 16.0	127.0 $\pm$ 15.3	126.9 $\pm$ 16.0	0.923
DBP (mmHg)	66.3 $\pm$ 9.4	65.8 $\pm$ 10.2	65.7 $\pm$ 9.6	0.850
Stent Types (%)				0.484
BMS	27.5	13.9	19.6	
DES	60.0	66.7	67.4	
Both	12.5	19.4	13.0	
Medications (%)				
Aspirin	90.3	94.3	96.4	0.3456
Thienopyridine	62.4	58.5	78.2	0.654
Statin	73.1	66.0	61.8	0.338
ACEI/ARB	63.4	66.0	56.4	0.553

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMS, bare-metal stent; DES, drug-eluting stent; ISR, in-stent restenosis. Values are the mean $\pm$ s.d. *P* values were obtained using the Kruskal–Wallis test for continuous variables and the χ^2 -test for categorical variables.

types of medications they were prescribed, among those groups (Table 1). Original stent types did not vary between the ISR group and the no lesion group as shown in Table 1. The percentage of patients with diabetes mellitus seemed higher in the de novo lesion group but no statistical significance was found.

The LDL-C level of patients with a past history of CAD was lowered to the target level (<100 mg/dL) in accordance with the guideline of the Japan Atherosclerosis Society for secondary prevention of CAD, while HDL-C levels in the CAD group were still lower than those of the non-CAD group (LDL-C, 95.7 ± 23.8 vs. 114.1 ± 31.8 , $p < 0.001$; HDL-C, 47.2 ± 12.1 vs. 55.6 ± 16.0 , $p < 0.001$, respectively). On the other hand, in the CAD group, serum lipid parameters were not related to whether restenosis or de novo lesions were present, indicating that quantitative changes in lipid profile are not sufficient to predict a recurrence of CAD (Table 2). Levels of hsCRP also had no relationship with the presence or absence of coronary lesions in the CAD group. On the other hand, although not statistically significant, HbA1c tended to be larger in the former than in latter (Table 2).

3.2. Serum MPO/PON1 ratio is an independent risk indicator of the recurrence of coronary artery disease

We next focused on MPO and PON1 that potentially represent functional qualities of plasma lipids and lipoproteins. First, we compared those markers between patients with and without a past history of CAD. There was no significant difference in serum MPO levels between CAD and non-CAD groups (207.1 (158.7 – 255.5) ng/mL vs. 237.8 (202.6 – 273.0) ng/mL, $p = 0.324$), whereas PON1 activity as paraoxonase was significantly lower in the CAD group than in the non-CAD group (220.0 (209.7 – 230.3) U/L vs. 251.1 (237.5 – 264.8) U/L, $p < 0.001$). Linear regression analysis demonstrated weak but significant inverse correlations of PON1 paraoxonase activity with age ($r^2 = 0.085$, $p < 0.001$) and the rate of concurrent diabetes mellitus ($r^2 = 0.049$, $p < 0.001$). Age and the percentage of patients with diabetes mellitus at baseline differed significantly between the non-CAD and CAD groups (age, 60.2 ± 13.9 years vs. 67.6 ± 10.0 years, $P < 0.001$; diabetes mellitus, 15.7% vs 51.5% , $P < 0.001$, respectively), indicating that those factors might affect the decrease in PON1 paraoxonase activity in CAD patients.

As shown in Table 2, Kruskal–Wallis test indicated significant differences of serum MPO mass and PON1 paraoxonase activity among CAD patients depending on whether they had coronary lesion at CAG re-examination. We also evaluated arylesterase activity to elucidate whether PON1 enzyme activity is independent of paraoxonase polymorphisms. Significant differences in arylesterase activity among no lesion, de novo lesion, and ISR groups were observed as well (101.0 ± 21.5 U/L vs. 87.0 ± 15.0 U/L vs. 87.1 ± 16.2 U/L, respectively, $P = 0.006$).

When we evaluated the relative effect of these parameters by calculating serum MPO mass/PON1 paraoxonase activity (MPO/PON1 ratio), there was no significant difference in MPO/PON1 ratio between the non-CAD and CAD groups (1.11 (0.93 – 1.30) vs. 1.20 (0.80 – 1.60), $p = 0.731$). In contrast, MPO/PON1 ratio was significantly different among CAD patients depending on the recurrence of coronary lesion (Table 2). We investigated if co-assessment of PON1 improves the diagnostic accuracy of MPO in CAD risk stratification by assessing NRI and IDI. The category-free NRI of MPO/PON1 ratio for CAD risk stratification was greater than that of MPO alone (39.3% vs 34.7% , $p = 0.023$ and 0.040 , respectively). The IDI after addition of MPO/PON1 ratio to the established risk factors (gender, age, smoking, hypertension, diabetes mellitus and dyslipidemia) was also large compared with that of MPO alone (10.4% vs. 8.8% , $p < 0.001$ in both). In multivariate logistic regression analysis that adjusted for age, gender, history of smoking, hypertension,

Table 2

Comparisons of laboratory parameters among the CAD patients.

	No lesion (n = 74)	De novo (n = 40)	ISR (n = 44)	P-value
T-Cho, mg/dL	163.9 ± 28.3	166.0 ± 29.6	167.9 ± 30.3	0.787
LDL-C, mg/dL	94.4 ± 24.5	96.0 ± 25.1	97.5 ± 21.4	0.689
HDL-C, mg/dL	47.0 ± 12.4	46.5 ± 10.5	48.4 ± 13.2	0.532
TG, mg/dL	133.7 ± 69.7	132.3 ± 71.8	129.2 ± 69.4	0.886
non HDL, mg/dL	117.0 ± 27.2	119.5 ± 28.2	119.5 ± 27.4	0.894
apo-AI, mg/dL	131.5 ± 23.9	130.8 ± 20.7	130.6 ± 23.0	0.990
apo-AII, mg/dL	32.5 ± 6.4	31.3 ± 5.8	31.5 ± 6.3	0.371
apo-B, mg/dL	72.4 ± 14.8	73.3 ± 16.2	73.7 ± 14.4	0.920
apo-CII, mg/dL	4.2 ± 1.5	4.3 ± 1.8	4.5 ± 2.0	0.605
apo-CIII, mg/dL	10.6 ± 3.8	10.7 ± 4.3	10.8 ± 3.8	0.908
apo-E, mg/dL	3.9 ± 1.0	3.8 ± 1.0	3.8 ± 1.0	0.489
Glu, mg/dL	103.4 ± 27.6	111.7 ± 36.0	107.8 ± 32.3	0.465
HbA1c, %	5.8 ± 0.7	6.3 ± 1.4	6.3 ± 1.3	0.075
hsCRP, mg/dL	0.14 ± 0.22	0.12 ± 0.14	0.10 ± 0.12	0.639
MPO mass, ng/mL	123.6 (101.2–145.9)	266.0 (134.1–397.8)	294.0 (174.8–413.2)	0.021
PON1 activity, U/L	234.3 (219.4–249.3)	200.6 (182.9–218.4)	213.6 (191.3–235.8)	0.009
MPO/PON1 ratio	0.56 (0.46–0.66)	1.79 (0.67–2.91)	1.77 (0.73–2.81)	0.002

Abbreviations: ISR, in-stent restenosis; T-Cho, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; apo, apolipoprotein; Glu, glucose; Hb, hemoglobin; hsCRP, high sensitivity C-reactive protein; MPO, myeloperoxidase; PON, paraoxonase. Values are the mean ± s.d. or geometric mean (95% confidence interval).

P values were obtained using Kruskal–Wallis test.

dyslipidemia, and diabetes mellitus, elevated MPO/PON1 ratio was associated with the prevalence of coronary lesion (ISR or de novo lesion) in the CAD group (OR 2.89, 95% CI 1.36–6.12, $P = 0.006$). The prognostic cutoff for MPO/PON1 ratio was set according to the ROC curve at 1.59 (see [Supplementary Appendix](#)), with sensitivity, specificity, and overall accuracy of 25.4%, 98.6%, and 64.4%, respectively. The area under the curve was 0.662 (95% CI 0.570–0.753, $p < 0.001$). Logistic regression analysis, adjusted for the factors indicated above, revealed that MPO/PON1 ratio >1.59 was independently associated with incidence of both ISR and de novo lesion as shown in [Table 3](#).

3.3. MPO/PON1 ratio represents anti-inflammatory function and cholesterol efflux capacity of HDL

Meanwhile, we also investigated whether serum MPO/PON1 ratio is associated with the HDL properties using ex vivo systems. First, a relationship between MPO/PON1 ratio and anti-

inflammatory function of HDL was examined. Complementary cell culture experiments revealed that TNF α markedly increased VCAM-1 expression in HUVEC, and the cytokine-induced VCAM-1 expression was significantly attenuated by pretreatment of the cell with HDL from CAD patients ([Fig. 2A](#)). Interestingly, the inhibitory effect of HDL was greater with low MPO/PON1 ratio than that with high MPO/PON1 ratio ([Fig. 1A](#)). Such relationships were held among non-CAD patients ([Fig. 1B](#)).

Next, to assess the impact of MPO/PON1 ratio on cholesterol efflux from macrophage, ex vivo efflux experiments were performed. Cholesterol efflux capacity of apolipoprotein B-depleted serum was reduced by approximately 6.3% in patients with MPO/PON1 ratio >3 compared patients with MPO/PON1 ratio <1 (MPO/PON1 <1 group vs. MPO/PON1 >3 group, $25.6 \pm 8.4\%$ vs. $19.3 \pm 5.1\%$, respectively, $p < 0.05$, [Fig. 2](#)).

4. Discussion

It has been postulated that MPO acts on LDL and HDL to cause chemical and oxidative modifications of the lipid and protein component [6]. In contrast, PON1 potentially protects LDL and HDL from oxidative stress [8]. Thus, it seems reasonable that MPO/PON1 ratio represents the function of lipoproteins. The present study demonstrated that MPO/PON1 ratio was independently associated with both restenosis and recurrent CAD, while lipid profile and other conventional risk factors did not show such relationships. Moreover, the HDL isolated from patients with high MPO/PON1 ratio exhibited attenuated anti-inflammatory properties and impairment of cholesterol efflux capacity. This is the first report that documented a direct correlation between MPO/PON1 ratio and function of HDL.

Two excellent works have previously demonstrated that MPO is a valuable predictor for both new-onset CAD and cardiovascular mortality [14,15]. Both the category-free NRI and IDI, novel methods for evaluating improvement in risk discrimination [13], of MPO/PON1 ratio for CAD risk stratification was greater than that of MPO alone, indicating that combination of PON1 with MPO offers greater improvement in CAD risk discrimination than single application of MPO.

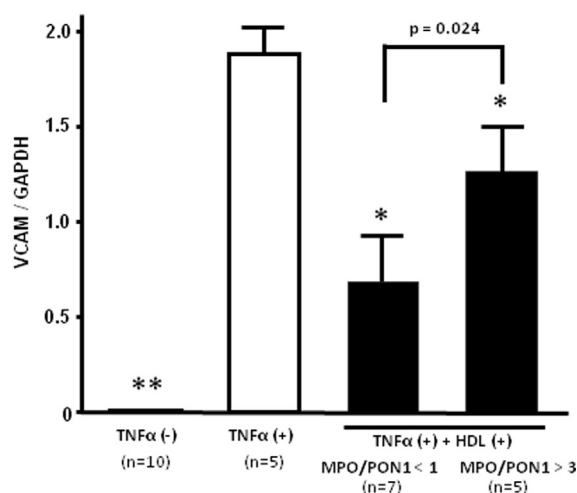
Table 3

Multiple logistic regression analysis for independent determinants of de novo lesion and ISR occurrence.

	OR	95% CI	P-value
A. De novo lesion			
MPO/PON1 ratio >1.59	3.48	1.29–9.40	0.014
Gender	2.65	0.88–8.01	0.083
Age	1.01	0.97–1.05	0.724
Smoking	0.98	0.41–2.35	0.969
Hypertension	1.11	0.36–3.46	0.856
Diabetes mellitus	1.91	0.90–4.05	0.093
Dyslipidemia	0.69	0.27–1.76	0.433
B. ISR			
MPO/PON1 ratio >1.59	6.44	2.15–19.32	0.001
Gender	3.50	1.10–11.1	0.063
Age	1.00	0.96–1.04	0.988
Smoking	0.40	0.15–1.05	0.063
Hypertension	2.89	0.77–10.87	0.116
Diabetes mellitus	1.95	0.89–4.28	0.095
Dyslipidemia	0.86	0.32–2.33	0.764

Abbreviations: ISR, in-stent restenosis; OR, odds ratio; CI, confidence interval; MPO, myeloperoxidase; PON, paraoxonase.

A CAD group



B Non CAD group

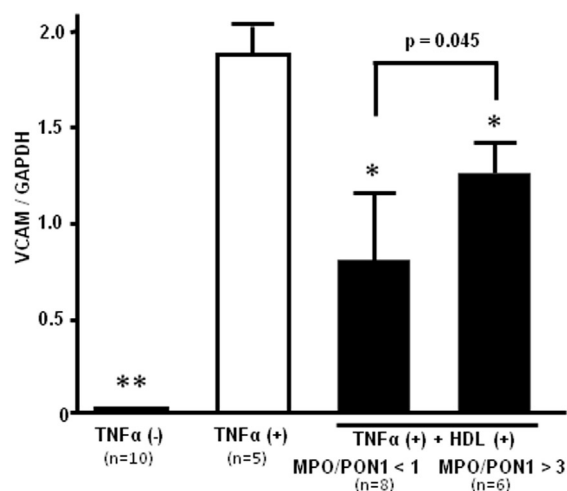


Fig. 1. Inhibition of tumor necrosis factor (TNF)- α induced vascular cell adhesion molecule (VCAM)-1 by high-density lipoprotein (HDL). Human umbilical vein endothelial cells were incubated with or without HDL for 16 h, and then stimulated with or without 10 ng/ml of TNF α for 6 h. HDL was purified from patient's serum with myeloperoxidase/paraoxonase 1 (MPO/PON1) ratio <1 or >3. The expression of VCAM1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained by real-time polymerase chain reaction and the value of VCAM was standardized by GAPDH. (A) Comparisons among CAD patients. (B) Comparisons among non CAD patients. *P* values were obtained using analysis of variance and Scheffe's multiple-comparison *t* test. **p* < 0.05 and *p* < 0.01 vs. TNF α stimulation without HDL pretreatment.

On the other hand, some studies did not document a positive associations between MPO and CAD [16]. The reasons for these contradicting results are speculative, but assay condition in the measurement would have influenced the outcomes. Most previous studies utilized MPO mass instead of its activity, as is the case with this study. Although strong correlations between MPO mass and activity have been reported [16], it remains controversial which reflects the accurate in vivo bioactivity of MPO. Enzymatic activity of MPO depends on local concentration of its co-substrate, hydrogen peroxide (H₂O₂), in vivo [17]. Since MPO activity is measured under saturated amounts of substrate, the ex vivo measurement of serum MPO activity may not always reflect the in vivo activity.

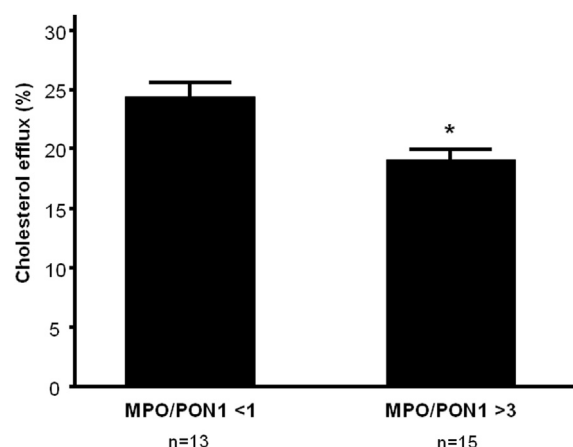


Fig. 2. Effect of MPO/PON1 ratio on HDL efflux capacity. THP-1 cells were differentiated into macrophages and loaded with [³H]-cholesterol and TO-901317. After incubation for 4 h with 2% apolipoprotein B-deficient serum, cholesterol efflux was measured (*n* = 13 for patients with MPO/PON1 <1 and *n* = 15 for patients with MPO/PON1 >3). Values represent mean $\pm$ S.D. *Indicates statistically significant differences from patients with MPO/PON1 <1 (*P* < 0.05) by Student's *t*-test.

A previous study by Inoue et al. showed that PON1 enzyme activities in diabetic patients were significantly lower than those in controls, while there were no differences in serum PON1 concentrations between patients and controls [18]. Moreover, it has been reported that genetic polymorphism 192Q/R affects paraoxonase activity, but not arylesterase activity [18]. Therefore, we assessed PON1 activity instead of mass in the present study, and demonstrated that both paraoxonase and arylesterase activities were associated with the prevalence of coronary lesions in the CAD group, indicating that the results are independent of the genetic polymorphism.

Although the pathogenesis of atherosclerosis and that of restenosis are presumably different, the high MPO/PON1 ratio was independently correlated with not only occurrence of non-target coronary lesions but also ISR. Growing evidence indicates that oxidative stress plays an important role in the formation of hypertrophic neointima through promoting vascular smooth muscle cell proliferation [19]. Angioplasty induces vascular inflammation [20] and up-regulates NAD(P)H oxidase in the vessel wall, followed by superoxide production that can form H₂O₂ [21]. It has been shown that MPO is enriched in human atherosclerotic lesions [22]. In the presence of H₂O₂ and chloride, MPO generates the potent oxidant hypochlorous acid (HOCl), that can induce endothelial nitric oxide synthase (eNOS) uncoupling, whereby eNOS turns into superoxide-producing enzyme [23]. Thus, there is a possibility that MPO is involved in the development of restenosis via induction of oxidative stress. On the contrary, Demirbag et al. have recently reported that ISR was negatively correlated with paraoxonase and arylesterase activities in patients with bare-metal stents, suggesting that anti-inflammatory and anti-oxidative effects of PON1 might contribute to alleviation of restenosis [24].

Recently, Khera et al. have shown that HDL from CAD patients showed less capacity in promoting cholesterol efflux compared to that from health controls and the efflux capacity of HDL is a potent determinant of cardiovascular events [25]. Meanwhile, Patel et al. demonstrated that HDL has less anti-oxidative capacity in patients with acute coronary syndrome compared with non-CAD or stable CAD patients [26]. Thus, accumulating evidence indicates that dysfunctional HDL could be a novel therapeutic target for CAD. However, conventional methods to assess functional changes in lipoproteins have not been established for clinical use. MPO

generates not only atherogenic LDL but also dysfunctional HDL particles [6]. Recent studies demonstrate that MPO modifies apoA-I and reduces its anti-inflammatory and anti-oxidative properties [7]. Huang et al. have shown that HOCl produced by MPO targets Trp72 of apoA-I and impairs ATP-binding cassette transporter A1-dependent cholesterol transport [27]. In contrast, the decreased PON1 activity is likely further to inhibit the anti-atherosclerotic properties of HDL or apoA-I [28]. These lines of evidence support our notion that high MPO with low PON1 activity could be a substitute marker for dysfunctional HDL.

In the present study, HDL-C levels in CAD group were lower than in non-CAD group. However, there was no significant difference in MPO/PON1 ratio between these groups.

Since this was just a comparison between the presence and absence of past CAD history, one plausible explanation is that non-CAD group might have included patients with high risk of CAD and vice versa. On the other hand, MPO/PON1 ratio was significantly different among CAD patients depending on the prevalence of coronary lesion recurrence, while HDL-C levels did not show such relationship. These findings suggest that quantity of HDL alone is not sufficient for CAD risk stratification, and patients with well-preserved HDL property may have less risk for CAD than those with dysfunctional HDL, even if their HDL-C levels were low.

In conclusion, the present findings suggest that serum MPO mass and PON1 activity as well as MPO/PON1 ratio could be useful markers for CAD risk stratification through modulation of HDL properties.

5. Study limitations

There are several limitations in this study. First, the present study is exploratory because the number of participants was small. In addition, since serum samples were obtained when patients underwent CAG re-examination, the present findings alone are insufficient to draw a conclusion that MPO/PON1 ratio is a predictor of CAD. Therefore, large prospective randomized studies are further required to elucidate the precise role of MPO and PON1 in CAD risk stratification. Second, we measured MPO concentration in serum but not in plasma, while most previous studies assessed plasma MPO concentration. Since PON1 is calcium-dependent, and therefore EDTA sensitive, we had to use serum as a sample material. It should be noted that MPO concentrations in serum are generally higher than in plasma because clot formation activates granulocytes to release MPO [16]. Third, there is a possibility that MPO might promote the progression of atherosclerosis through modulation of LDL function. Recently, Boudjeltia et al. have developed the ELISA system to measure circulating levels of LDL modified by the MPO-H₂O₂-chloride system, commonly named Mox-LDL [29]. This sort of method may provide a better understanding of this issue. In addition, serum PON1 activity does not entirely reflect HDL-associated PON1 activity alone, although it has been reported that PON1 exhibited higher activity when it binds with HDL than when it is free, and HDL phospholipids affect PON1 catalytic and biological activities [30]. Fourth, it is possible that medications might influence serum MPO/PON1 ratio and/or HDL property. In this study, neither of them showed any correlation with patient's medications including statins (data not shown). However, prospective interventional trial is needed to elucidate this issue as well.

Conflict of interest

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.atherosclerosis.2014.03.009>.

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