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# The relationship between unique gut microbiome-derived lipid metabolites and subsequent revascularization in patients who underwent percutaneous coronary intervention

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**Abstract**

**Background and aims:**

Studies have recently revealed the linoleic acid metabolic pathway of *Lactobacillus plantarum*, the representative gut bacterium in human gastrointestinal tract, and anti-inflammatory effects of the metabolites in this pathway. However, no clinical trials have evaluated the association between these metabolites and revascularization in patients who underwent percutaneous coronary intervention (PCI).

**Methods:**

We retrospectively reviewed patients who underwent PCI with subsequent revascularization or coronary angiography (CAG) without revascularization. Patients with frozen blood samples at the index PCI and revascularization or follow-up CAG were enrolled.

**Results**:

Among 701 consecutive patients who underwent PCI, we enrolled 53 patients who underwent subsequent revascularization and 161 patients who underwent follow-up CAG without revascularization. Patients who underwent revascularization showed significantly lower plasma 10-oxo-octadecanoic acid (KetoB) levels (720.5 [551.6–876.5] vs. 818.4 [641.1–1103.6 pg/mL]; p=0.01) at index PCI. Multivariate logistic regression analysis revealed that decreased plasma KetoB levels at the index PCI were independently associated with subsequent revascularization after PCI (odds ratio; 0.90 per 100 pg/mL increase, 95% confidence interval; 0.82–0.98). Additionally, in vitro experiments showed that the addition of purified KetoB suppressed the mRNA levels of IL-6 and IL-1ß in macrophages and IL-1ß mRNA in neutrophils.

**Conclusions:**

Plasma KetoB level at the index PCI was independently associated with subsequent revascularization after PCI, and KetoB could act as an anti-inflammatory lipid mediator in

macrophages and neutrophils. The assessment of gut microbiome-derived metabolites may help predict revascularization after PCI.

## 1. Introduction

Knowledge of the gut microbiome has expanded recently owing to next-generation sequencing techniques. Consequently, the relationship between the human gut microbiome and various host disorders has become clear. Previous studies have reported a higher presence of coronary artery disease (CAD) in patients with specific enterotypes of the gut microbiome[1, 2]. Generally, the individual gut microbiome is known to be resilient and remain stable in long-term[3], and several specific gut bacteria are associated with CAD[4], suggesting that individual gut microbiome features could be a potential predictor for the development of CAD. In addition, the gut microbiome generates several metabolites that have physiological effects on the host. For instance, trimethylamine *N*-oxide (TMAO) is widely known to be a risk factor for atherosclerosis[5-7]. Butyrate from the gut microbiome has anti-inflammatory effects that may suppress atherogenesis[8].

In a recent study, the metabolic pathway of linoleic acid in *Lactobacillus plantarum* became clear[9]. In this pathway, linoleic acid is converted to oleic acid via 10-hydroxy-cis-12-octadecenoic acid (HYA), 10-oxo-*cis*-12-octadecenoic acid (KetoA), 10-oxo-*trans*-11-octadecenoic acid (KetoC), 10-oxo-octadecenoic acid (KetoB), 10-hydroxy-octadecenoic acid (HYB). HYB and KetoB are also generated from oleic acid. Lactobacillus is among the most common bacteria in the human gastrointestinal tract, and linoleic and oleic acids are among the most highly consumed unsaturated fatty acid in the human diet. A recent report showed that the levels of the early metabolites of this pathway in the colon, small intestine, and plasma are notably lower in germ-free mice[9], showing that the generation of these lipid metabolites may depend not on the host but on the gut microbiome. Some in vitro studies have reported the physiological effects of metabolites in this pathway. All hydroxy and oxo fatty acids in this pathway activate proliferator-activated receptor (PPAR)α, and KetoA activates both PPARα and PPARγ[10]. Another study demonstrated that KetoC has antioxidant and anti-inflammatory

effects[11, 12]. In addition, HYA and KetoA have hypolipidemic effects[13], and HYA administration in mice improves glucose homeostasis[14].

These results indicate that the unique linoleic acid metabolites generated by the gut microbiome might suppress host atherogenesis. However, no clinical study has evaluated the effects of these metabolites on CAD development. In addition, the stability of these metabolites in human blood over time and their relationship with other patient characteristics remain unclear. Thus, in this study, we evaluated the relationship between linoleic acid metabolites in the gut microbiome and subsequent revascularization after percutaneous coronary intervention (PCI), their clinical features, and stability by examining serial blood samples of patients who underwent PCI. We conducted an in vitro experimental study to assess the potential physiological effects of these metabolites to investigate the potential mechanisms of this relationship.

## 2. Materials and Methods

### 2.1.1. A retrospective clinical investigation of patients who underwent PCI

This single-center retrospective clinical study used cryopreserved blood samples from patients who underwent PCI. At our institute, blood samples were obtained from patients who underwent coronary angiography (CAG) or PCI in a prospective registry to evaluate the relationship between several biomarkers and clinical outcomes in patients with CAD (UMIN ID: 000030297). The inclusion criteria of this study were patients who underwent PCI with ≥ second generation drug-eluting stents or drug-coated balloons between December 2014 and March 2019 and who underwent follow-up CAG with and without revascularization. Exclusion criteria were patients without frozen blood samples at the index PCI or follow-up (follow-up CAG or revascularization) and those undergoing hemodialysis. Indications for index PCI and revascularization were determined based on the severity of angiographical stenosis and

ischemia estimation with stress electrocardiogram, myocardial scintigraphy, invasive or non-invasive fractional flow reserve, and resting physiological indices.

We categorized the enrolled patients according to the presence (revascularization group) or absence (non-revascularization group) of subsequent revascularization after the index PCI and compared plasma lipid metabolite levels between the two groups. Revascularization was defined as any subsequent PCI, including revascularization for restenosis of the index PCI lesions and new or progressive lesions of the target and other vessels. Moreover, to clarify the stability over time and the clinical feature of these metabolites in all included patients, we compared the plasma levels of these metabolites at index PCI with those at follow-up CAG or revascularization. We also evaluated the relationship between metabolites and other patient characteristics at the index PCI.

The study protocol complied with the Declaration of Helsinki, and the Ethics Committee of Kobe University Hospital approved it. Informed consent was obtained as an opt-out from the Kobe University Graduate School of Medicine, Department of Cardiology website because the data were collected retrospectively.

**2.1.2. Blood sampling and measurement of metabolites**

Blood samples were obtained from patients who underwent CAG or PCI and were cryopreserved. Blood sampling was performed at the catheter laboratory immediately before the catheter procedure from the introducer sheath inserted into the radial, brachial, or femoral artery. Hence, we can retrospectively measure lipid metabolites using liquid chromatography-mass spectrometry from these secure blood samples.

Measurement of plasma lipid metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed as previously described[15, 16]. The system consisted of a Q-Trap 6500 system (Sciex) equipped with a Shimadzu LC-30AD HPLC system. A

ZORBAX Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 μm, Agilent Technologies, Santa Clara, CA, United States) was used with a methanol/water/acetic acid gradient of 55:45:0.01 to 98:2:0.01 (v/v/v) at a 0.4 ml/min flow rate. A multiple-reaction monitoring method was developed with signature ion pairs Q1 (parent ion)/Q3 (characteristic fragment ion) for each molecule to monitor and quantify the levels of eicosanoids. Identification was conducted according to published criteria using the LC retention time, specific fragmentation patterns, and at least six diagnostic fragmentation ions. Quantification was performed based on the peak area of the multiple reaction monitoring chromatograph, and linear calibration curves were obtained with authentic standards for each compound. This study focuses on hydroxy- and oxo-fatty acids in the linoleic acid metabolic pathway. We measured the plasma levels of HYA, HYB, 10-hydroxy-*trans*-11-octadecenoic acid (HYC), KetoA, and KetoB. Since HYA and HYC cannot be classified by mass spectrometry because of similar elution peaks, we measured the sum of HYA and HYC in this analysis. Additionally, because the definitive elution peak of KetoC could not be identified, we could not measure plasma KetoC levels.

### 2.2. In vitro experimental study with mRNA analysis by adding purified lipid metabolites to experimental macrophages and neutrophils

We performed mRNA analysis using macrophages and neutrophils to assess the anti-atherogenic effects of lipid metabolites. RAW 264.7, HL-60, and EA.hy 926 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Riken BRC (BioResource Research Center, JP). RAW264.7 and EA.hy 926 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM), HL-60 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 with 1μM all-trans retinoic acid from Wako (Wako Pure Chemical Industries Ltd., Osaka, JP). All media were purchased from Wako and supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA).

Kishino and Ogawa provided standards for HYA, HYB, HYC, KetoA, KetoB, and KetoC. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared from 1 μg of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Kyoto, Japan). Real-time polymerase chain reaction (real-time PCR) was performed with TB Green Premix Ex Taq II (Takara). Primers were obtained from Takara Bio Inc. Amplification reactions were performed in duplicate using a LightCycler 96 Real-Time PCR system (Roche Molecular Systems, CA, USA), and fluorescence curves were analyzed using the included software. 18S ribosomal RNA was used as an internal control. Relative quantification was performed using the ΔΔCt method.

### 2.3. Statistical analysis

Baseline categorical variables were reported as percentages, and continuous variables were reported as mean ± SD or as median and interquartile if the data were not normally distributed. For discrete variables, comparisons were performed using chi-square analysis or Fisher's exact test. For continuous variables, comparisons were performed using a 2-tailed, unpaired t-test, Welch test, or Wilcoxon test, according to the data of normal or non-normal distribution and equal variance, respectively. Logistic regression analysis was performed to identify independent factors associated with revascularization. To compare the plasma levels of metabolites between the index PCI and follow-up, we performed a paired t-test. Spearman's rank correlation was used to assess the relationship between the plasma metabolite levels and other laboratory findings. Net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were used to assess the contribution of plasma KetoB levels. Statistical analyses were performed using SPSS for Windows, version 28 (IBM SPSS Inc., Chicago, IL, USA). R software version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria) with the PredictABEL package[17] was used to calculate the NRI and IDI.

## 3. Results

### 3.1. Study population

Among 701 consecutive patients, we enrolled 53 patients who underwent revascularization (revascularization group) and 161 patients who underwent follow-up CAG without revascularization (non-revascularization group) (see Supplementary Appendix). The median duration between index PCI and the second coronary angiography was 301.0 [265.75–379.75] days. The period tended to be longer in the revascularization group than in the non-revascularization group (339.0 [259.5–530.0] vs. 298.0 [266.0–355.5 days]; p=0.07). Among the 214 patients, 35 underwent follow-up CAG because of chest symptoms, 3 underwent follow-up CAG because of positive non-invasive imaging tests, and the remaining 176 underwent follow-up CAG without significant symptoms as routine clinical practice. Subsequently, 53 patients (56 lesions), including 4 acute coronary syndromes, underwent revascularization (revascularization group), while the remaining 161 patients did not undergo revascularization (non-revascularization group). Among 56 lesions underwent revascularization, 42 lesions underwent PCI due to severe angiographic stenosis (%DS >90%), and the remaining 14 lesions were treated due to ischemia upon positive myocardial scintigraphy and/or invasive fractional flow reserve and/or resting physiological indices. Regarding to the revascularization lesion, 20 were treated for target lesions of index PCI (18 in-stent restenosis), 17 were treated for non-target lesions in target vessels, and 19 were treated for lesions in non-target vessels.

### 3.2. Comparison of patients' characteristics between the revascularization and non-revascularization groups

The baseline characteristics and laboratory data of the study participants, plasma lipid

metabolite levels at the index PCI and follow-up, and changes in lipid metabolite values during follow-up are shown in Table 1. The patients in the revascularization group had a significantly higher prevalence of diabetes mellitus (60.4% vs. 40.4%; p=0.009), higher serum HbA1c level (6.63 ± 1.16 vs. 6.30 ± 0 .93%; p=0.03), and lower serum HDL cholesterol level (42.7 ± 9.6 vs. 47.6 ± 12.5 mg/dL; p=0.02) than those in the non-revascularization group. Regarding the lipid metabolites, patients in the revascularization group had significantly lower plasma KetoA levels (244.6 [149.2–315.0] vs. 291.6 [203.9–393.1 pg/mL]; p=0.03) and KetoB levels (720.5 [551.6–876.5] vs. 818.4 [641.1–1103.6 pg/mL]; p=0.01) at the index PCI than those in the non-revascularization group. LDL cholesterol levels, triglyceride levels, and the prevalence of other traditional cardiovascular risk factors were not different between the two groups. At follow-up, plasma KetoB levels were numerically lower in the revascularization group than in the non-revascularization group, although the difference was not statistically significant (727.1 [473.1–917.0] vs. 790.4 [533.9–1095.1 pg/mL]; p=0.11). In contrast, there was no significant difference between the two groups in the absolute change values of lipid metabolites during the follow-up period.

Multivariate logistic regression analysis revealed that decreased plasma KetoB level at the index PCI (odds ratio [OR]; 0.90 per 100 pg/mL increase, 95% confidence interval [CI]; 0.82–0.98), presence of diabetes mellitus (OR; 2.05, 95% CI; 1.05–3.98), and lower serum HDL cholesterol level (OR, 0.97; 95% CI; 0.94–1.00) were independently associated with subsequent revascularization after PCI (Table 2). When plasma KetoB level at index PCI was added to the eight traditional risk factors (age, male sex, prior CAD, multivessel disease, presence of DM, serum HDL and LDL cholesterol level, triglyceride level), incremental reclassification ability to predict revascularization after PCI, as evaluated by net reclassification improvement (NRI) and integrated discrimination improvement (IDI), showed further increases (NRI=0.47, 95% CI 0.13–0.81, p=0.006; IDI=0.12, 95% CI 0.06–0.17, p

<0.001).

### 3.3. Change values of plasma KetoB levels during the follow-up and relationship between plasma KetoB levels and patients' characteristics in overall subjects

Laboratory data, plasma lipid metabolite levels at baseline and follow-up in all patients, mean change value, 95% CI, standard error, and p-value from a paired t-test are shown in Table 3. All plasma lipid metabolites showed no significant change from the index PCI to follow-up. In contrast, serum triglyceride and LDL cholesterol levels were significantly decreased. Median plasma KetoB levels changed from 780.7 (604.3–1026.6) to 769.1 (518.3–1075.5 pg/mL), and the mean change value of each patient was -28.8 pg/mL (95% CI: -131.6–73.9 pg/mL, standard error: 52.1).

The relationship between plasma KetoB levels and other patient characteristics (sex, age, BMI, HbA1c levels, serum triglyceride, HDL cholesterol, and LDL cholesterol levels) of all included patients are shown in Figure 1. In this study population, there was no significant difference in plasma KetoB levels between males and females (778.3 [616.2–1093.6] vs. 787.1 [553.9–989.8 pg/mL]; p=0.47). Plasma KetoB levels were not correlated with traditional cardiovascular risk factors such as age, BMI, HbA1c levels, and serum lipid profile levels. Similarly, the other plasma lipid metabolite levels showed no significant difference between males and females; KetoA (280.6 [186.5 –382.9] vs. 263.7 [198.1–368.2 pg/mL]; p=0.63), the sum of HYA and HYC (304.0 [168.6–745.6] vs. 288.8 [187.0–562.0 pg/mL]; p=0.76) and HYB (610.5 [406.2–891.2] vs. 564.7 [383.5–869.3 pg/mL]; p=0.57). In addition, plasma KetoA levels showed a statistically significant but weak negative correlation with serum LDL cholesterol levels (r=-0.017, p=0.02). In addition, the sum of HYA and HYC levels showed a significant but weak positive correlation with age (r=0.20, p=0.003) and plasma HYB levels (r=0.17, p=0.01). The other five factors showed no significant correlation with lipid metabolites

(see Supplementary Appendix).

### 3.4. KetoB shows anti-inflammatory properties in macrophages and neutrophils

Since plasma KetoB levels could be inversely correlated with CAD progression, we sought to investigate the biological functions of KetoB in macrophages and neutrophils. A murine macrophage cell line, RAW 264.7, was incubated with Kdo2 (0.5 μg/mL) for 1 h, then added 10 μM of HYA, KetoA, and KetoB for 1 h at 37 °C. IL-1ß mRNA levels were significantly reduced by adding KetoB and KetoA (Figure 2A). IL-6 mRNA levels were significantly reduced by adding KetoB, KetoA, or HYA (Figure 2B). Next, a human neutrophil cell line, HL-60, was incubated with Kdo2 (0.5μg/mL) for 1 h, followed by adding 10 μM of HYA, KetoA, and KetoB for 2 h at 37 °C. IL-1ß mRNA levels were significantly reduced by adding KetoB and KetoA (Figure 2E).

## 4. Discussion

In this retrospective clinical study, we demonstrated that decreased plasma KetoB levels at index PCI were independently associated with subsequent revascularization in patients who underwent PCI with ≥ second generation drug-eluting stents or drug-coated balloons. In addition, in all included patients, plasma KetoB levels showed very minimum correlation with other patient characteristics. Plasma KetoB levels showed no significant changes during the follow-up period (median follow-up period: 301.0 [265.75–379.75] days), showing its potential stability in vivo. Furthermore, in the in vitro cell experiment, KetoA and KetoB showed significant suppression of IL-1β mRNA expression in macrophages and neutrophils and IL-6 mRNA expression in neutrophils. These results indicate that KetoA and KetoB have anti-inflammatory properties in macrophages and neutrophils.

Previously, a specific enterotype of the gut microbiome classified by genomic analysis

was reported to be related to host CAD presence[1, 2]. However, the reason for contributing the types of gut microbiome patterns in the presence of CAD remains unclear. Recently, several studies have implied that the physiological effects of gut microbiome-derived metabolites may explain the relationship between specific enterotypes and host CAD. For instance, it has become evident that TMAO, a metabolite of trimethylamine generated by the gut microbiome, can promote atherogenesis because of the acceleration of vascular inflammation, endothelial dysfunction, atherothrombosis, and impairment of cholesterol metabolism[18]. In addition, several previous studies have shown that plasma TMAO concentration is a useful marker for the risk stratification of cardiovascular disease[5-7]. Thus, potential therapeutic strategies to lower TMAO levels are being discussed and developed to prevent CAD[18].

Recent in vitro and animal experiments have reported that metabolites in the linoleic acid metabolic pathway of the gut microbiome have physiological effects that suppress oxidation and inflammation[11, 12] and improve lipid and glucose metabolism[13, 14]. Based on previous data, we considered that these metabolites could inhibit atherogenesis and potentially predict revascularization after PCI. In this study, we demonstrated that, in addition to already known risk factors such as presence of diabetes mellitus and lower serum HDL cholesterol levels, decreased plasma KetoB levels were independently associated with revascularization after PCI. In addition, we demonstrated that adding plasma KetoB levels to the traditional risk factors increased the incremental reclassification ability to predict revascularization after PCI. These results indicate that measuring plasma KetoB levels is important for risk stratification in patients who underwent PCI. Although the classification of the gut microbiome by genomic analysis is certainly useful for risk stratification for CAD, these methods only classify patients into several types. However, identifying the metabolites that affect atherosclerosis enables more patient-specific risk stratification and may lead to new treatment strategies. This clinical study is the first on the relationship between linoleic acid lipid metabolites and

revascularization after PCI.

The detailed mechanisms of the relationship between increased plasma KetoB levels and increased incidence of revascularization remain unclear. However, we speculate that its anti-inflammatory properties might play a role. Previous studies have reported that KetoA activates PPARα and PPARγ, and other hydroxy and oxo-fatty metabolites, including KetoB, activate PPARα[10]. PPARα and PPARγ modulate atherosclerosis-associated inflammatory responses[19, 20]. Furthermore, our cell experiments demonstrated significant anti-inflammatory effects of KetoA and KetoB in suppressing IL-1β mRNA expression in macrophages and neutrophils and IL-6 mRNA expression in macrophages. Inflammation plays a prominent role in atherosclerosis development and destabilization[21, 22], and the contribution of macrophages and neutrophils to these processes has been firmly established[23, 24]. In addition, IL-1β is an inflammatory cytokine that directly leads to the development of CAD and induces the production of IL-6 in the downstream signaling pathway[25, 26]. Thus, we speculate that KetoB might reduce the incidence of revascularization after PCI owing to its suppressive effect on IL-1β and IL-6 mRNA expression in macrophages and neutrophils. Notably, Ridker et al. reported in a recent CANTOS trial that anti-inflammatory therapy with canakinumab, an antibody blocking IL-1β, suppresses MACE in patients with myocardial infarction[27]. These results support our hypothesis that suppressing IL-1β expression in macrophages and neutrophils in coronary vessel wall could inhibit atherogenesis and have important clinical implications for suppressing CAD development. However, it is unclear whether our cell experiment results can be adapted to in human coronary artery. To clarify in vivo anti-inflammatory effects, more studies are needed, such as evaluating the relationship between lipid metabolites and coronary sinus blood cytokine levels, or single cell RNA analysis of coronary plaque obtained by the directional coronary atherectomy. In present study, among 56 revascularization lesion, 20 lesions were restenosis of the target lesion of index PCI. In smooth muscle cell proliferation,

which is a major cause of in-stent restenosis, cytokines from macrophages and neutrophils play a significant role [28]. The effects of KetoA and KetoB to suppress IL-1β and IL-6 expression in macrophage and neutrophils can lead to the suppression of in-stent restenosis. However, our cell experimentation only clarify the partial effects related to the progression of atherosclerosis and restenosis after PCI. In previous studies, some metabolites generated from ω-3 polyunsaturated fatty acids were shown to have a effects to suppress the vascular smooth muscle cell proliferation and migration [29], and platelet activity [30]. The gut microbiome-derived lipid metabolites may also have such effects, and to evaluate other physiological effects of these metabolites are our future subject. Further studies should elucidate the mechanisms underlying the relationship between KetoB and revascularization after PCI and clarify whether KetoB could be a potential therapeutic target for preventing revascularization.

Another key finding of this study was that the plasma KetoB levels were stable over time. In this study, although serum triglyceride and LDL cholesterol levels significantly decreased during the follow-up period, plasma KetoB levels remained relatively unchanged. Furthermore, plasma KetoB levels at follow-up were lower in the revascularization group than in the non-revascularization group, although the difference was not statistically significant. These results emphasize the uniqueness of plasma KetoB level as a factor associated with revascularization after PCI, and one-time measurement of plasma KetoB levels might be useful for considering the risks of revascularization. There has been no study to date that has investigated serial changes in linoleic acid metabolites over time. Our results indicated that, in contrast to significant changes in lipid profile, plasma KetoB levels were relatively stable, showing the potential usefulness of one-time measurement of plasma KetoB levels as risk stratification for revascularization after PCI. A larger-scale study with longer-term follow-up is needed to clarify the precise changes over time and the clinical usefulness of plasma KetoB levels for long-term risk stratification after PCI.

Furthermore, this study revealed that there was slight correlation between patient characteristics and plasma levels of linoleic acid metabolites. These results indicate that these lipid metabolites are uniquely related to revascularization after PCI, independent of traditional CAD risk factors. However, previous studies reported that HYA and KetoA significantly decreased element-binding protein-1c mRNA expression in HepG2 cells[13]. The oral administration of KetoA decreased Srebp-1c, Scd-1, and Acc2 expression in the liver of mice fed a high-sucrose diet, showing that KetoA may have hypolipidemic effects[13]. In mice, HYA administration also reduced body weight, increased plasma GLP-1 levels, and suppressed increases in blood glucose in the oral glucose tolerance test[14]. These studies show that HYA and KetoA improve lipid profiles, HbA1c levels, and BMI, leading to the prevention of revascularization. Contrarily, although we could not separate HYA and HYC rigidly, the sum of HYA and HYC levels was not significantly different between the revascularization and non-revascularization groups. Furthermore, we could not find strong correlations between HYA and HYC, KetoA and serum lipid profiles, HbA1c levels, and BMI. Although still speculative, we consider that this conflict could be attributed to the difference in patient backgrounds between previous studies and ours. In this study, most patients had well-controlled dyslipidemia and DM at the index PCI. The mean serum LDL cholesterol level at index PCI was 99.7 ± 2.3 mg/dL, 167 of 214 patients (78% of enrolled patients) had been under statin therapy, and baseline serum HbA1c levels in patients with diabetes were 7.09 ± 0.11%. Thus, we currently consider that the impact of these lipid metabolites on lipid and glucose metabolism might be attenuated in patients with well-controlled dyslipidemia and DM, while it might become evident in the general population. Such speculation could partly explain the discordant results between our study and previous studies. Further clinical studies are needed to clarify the potential relationship between baseline lipid and diabetic profiles and the impact of lipid metabolites on lipid and glucose metabolism.

Our study has several limitations. First, this was a single-center retrospective study with limited sample size. The treatment for known CVD risk factors depended on the physician, and several unknown risk factors might have affected the results because of the retrospective nature of the study design. Furthermore, lifestyle information, such as dietary patterns and exercise habits, was lacking. Second, the linoleic acid metabolic pathway focused on in this study was shown to be gut microbiome-specific in mice and is speculated to be similar in humans, however, no studies have clearly demonstrated the specificity in humans. However, it should be mentioned that the homologous genes of the enzyme in the gut microbiome linoleic acid metabolic pathway were not detected in humans using the NCBI Basic Local Alignment Search Tool (BLAST) search. Third, HYA and HYC could not be categorized and measured as a total value, and the KetoC level could not be measured by mass spectrometry. Fourth, we enrolled patients with frozen blood samples at the index PCI, follow-up, and selection bias. Patients who underwent PCI without follow-up CAG were not included, and not all patients who underwent PCI and follow-up CAG had frozen blood samples. Fifth, the follow-up period from index PCI to follow-up CAG or revascularization was relatively short (median follow-up period: 301.0 [265.75–379.75] days). Therefore, we could not evaluate the long-term relationship between these metabolites and revascularization. A large-scale, prospective study with a longer follow-up period is necessary to confirm our results.

In conclusion, the Plasma KetoB levels at the index PCI were independently associated with subsequent revascularization after PCI, and the anti-inflammatory properties of KetoB could contribute to this relationship. Measuring plasma gut microbiome-derived lipid metabolite levels might help predict revascularization after PCI.

## Conflict of interest

This research received no specific financial support from any funding agency in the public, commercial, or not-for-profit sectors.

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## Author contributions

D. Fujimoto: conceptualization, methodology, data curation, interpretation, writing-original drafts. H. Otake, M. Shinohara: Conceptualization, methodology, writing-reviewing, and editing. H. Kawaori, T. Toba, S. Kakizaki, K. Nakamura, S. Sasaki, T. Hamana, H. Fujii, Y, Osumi: Conceptualization, methodology. N. Hayasaka: Investigation. S. Kishino, J Ogawa: resource, writing-reviewing, and editing. K. Hirata: Supervision. All authors have read and approved the final version of the manuscript.

## References


[1] Emoto, T, Yamashita, T, Sasaki, N, et al., Analysis of Gut Microbiota in Coronary Artery Disease Patients: a Possible Link between Gut Microbiota and Coronary Artery Disease, J Atheroscler Thromb, 2016;23:908-921.

[2] Emoto, T, Yamashita, T, Kobayashi, T, et al., Characterization of gut microbiota profiles in coronary artery disease patients using data mining analysis of terminal restriction fragment length polymorphism: gut microbiota could be a diagnostic marker of coronary artery disease, Heart Vessels, 2017;32:39-46.

[3] Faith, JJ, Guruge, JL, Charbonneau, M, et al., The long-term stability of the human gut microbiota, Science, 2013;341:1237439.

[4] Yoshida, N, Emoto, T, Yamashita, T, et al., Bacteroides vulgatus and Bacteroides dorei Reduce Gut Microbial Lipopolysaccharide Production and Inhibit Atherosclerosis, Circulation, 2018;138:2486-2498.

[5] Wang, Z, Klipfell, E, Bennett, BJ, et al., Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease, Nature, 2011;472:57-63.

[6] Koeth, RA, Wang, Z, Levison, BS, et al., Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis, Nat Med, 2013;19:576-585.

[7] Tang, WH, Wang, Z, Levison, BS, et al., Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk, N Engl J Med, 2013;368:1575-1584.

[8] Furusawa, Y, Obata, Y, Fukuda, S, et al., Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells, Nature, 2013;504:446-450.

[9] Kishino, S, Takeuchi, M, Park, SB, et al., Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition, Proc Natl Acad Sci U S A, 2013;110:17808-17813.

[10] Goto, T, Kim, YI, Furuzono, T, et al., 10-oxo-12(Z)-octadecenoic acid, a linoleic acid metabolite produced by gut lactic acid bacteria, potently activates PPARgamma and stimulates adipogenesis, Biochem Biophys Res Commun, 2015;459:597-603.

[11] Furumoto, H, Nanthirudjanar, T, Kume, T, et al., 10-Oxo-trans-11-octadecenoic acid generated from linoleic acid by a gut lactic acid bacterium Lactobacillus plantarum is cytoprotective against oxidative stress, Toxicol Appl Pharmacol, 2016;296:1-9.

[12] Yang, HE, Li, Y, Nishimura, A, et al., Synthesized enone fatty acids resembling metabolites from gut microbiota suppress macrophage-mediated inflammation in adipocytes, Mol Nutr Food Res, 2017;61.

[13] Nanthirudjanar, T, Furumoto, H, Zheng, J, et al., Gut Microbial Fatty Acid Metabolites Reduce Triacylglycerol Levels in Hepatocytes, Lipids, 2015;50:1093-1102.

[14] Miyamoto, J, Igarashi, M, Watanabe, K, et al., Gut microbiota confers host resistance to obesity by metabolizing dietary polyunsaturated fatty acids, Nat Commun, 2019;10:4007.

[15] Colas, RA, Shinohara, M, Dalli, J, et al., Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue, Am J Physiol Cell Physiol, 2014;307:C39-54.

[16] Tsuda, S, Shinohara, M, Oshita, T, et al., Novel mechanism of regulation of the 5-lipoxygenase/leukotriene B4 pathway by high-density lipoprotein in macrophages, Sci Rep, 2017;7:12989.

[17] Kundu, S, Aulchenko, YS, van Duijn, CM, et al., PredictABEL: an R package for the assessment of risk prediction models, Eur J Epidemiol, 2011;26:261-264.
[18] Yang, S, Li, X, Yang, F, et al., Gut Microbiota-Dependent Marker TMAO in Promoting Cardiovascular Disease: Inflammation Mechanism, Clinical Prognostic, and Potential as a Therapeutic Target, Front Pharmacol, 2019;10:1360.
[19] Zandbergen, F and Plutzky, J, PPARalpha in atherosclerosis and inflammation, Biochim Biophys Acta, 2007;1771:972-982.
[20] Chandra, M, Miriyala, S and Panchatcharam, M, PPARgamma and Its Role in Cardiovascular Diseases, PPAR Res, 2017;2017:6404638.
[21] Ross, R, Atherosclerosis--an inflammatory disease, N Engl J Med, 1999;340:115-126.
[22] Libby, P, Inflammation in atherosclerosis, Nature, 2002;420:868-874.
[23] Moore, KJ and Tabas, I, Macrophages in the pathogenesis of atherosclerosis, Cell, 2011;145:341-355.
[24] Soehnlein, O, Multiple roles for neutrophils in atherosclerosis, Circ Res, 2012;110:875-888.
[25] Dinarello, CA, Interleukin-1 in the pathogenesis and treatment of inflammatory diseases, Blood, 2011;117:3720-3732.
[26] Mai, W and Liao, Y, Targeting IL-1$\beta$ in the Treatment of Atherosclerosis, Frontiers in Immunology, 2020;11.
[27] Ridker, PM, Everett, BM, Thuren, T, et al., Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease, N Engl J Med, 2017;377:1119-1131.
[28] Welt, FG and Rogers, C, Inflammation and restenosis in the stent era, Arterioscler Thromb Vasc Biol, 2002;22:1769-1776.
[29] Akagi, D, Chen, M, Toy, R, et al., Systemic delivery of proresolving lipid mediators resolvin D2 and maresin 1 attenuates intimal hyperplasia in mice, FASEB J, 2015;29:2504-2513.
[30] Shinohara, M, Kibi, M, Riley, IR, et al., Cell-cell interactions and bronchoconstrictor eicosanoid reduction with inhaled carbon monoxide and resolvin D1, Am J Physiol Lung Cell Mol Physiol, 2014;307:L746-757.

**Keywords:** lipid metabolites；gut microbiome; enterobacteria; coronary artery disease; secondary prevention; linoleic acid, oleic acid

**Abbreviations:**

CAD, coronary artery disease

CAG, coronary angiography

CI, confidence interval

HbA1c, hemoglobin A1c

HDL, high-density lipoprotein

IDI, integrated discrimination improvement

LDL, low-density lipoprotein

NRI, net reclassification improvement

OR, odds ratio

PCI, percutaneous coronary intervention

PPAR, proliferator-activated receptor

TMAO, trimethylamine *N*-oxide

**Tables**

**Table1. Baseline patient characteristics, plasma lipid metabolite levels at follow-up, and change value during follow-up**

| | Revascularization (n=53) | Non-revascularization (n=161) | p-value |
|---|---|---|---|
| Age (yr) | 69.8 ± 11.2 | 71.2 ± 9.3 | 0.37 |
| Male, n (%) | 47 (88.7) | 125 (77.6) | 0.08 |
| Body mass index (kg/m$^2$) | 24.7 ± 3.9 | 24.4 ± 3.9 | 0.57 |
| Current smoking, n (%) | 11 (20.8) | 24 (14.9) | 0.32 |
| Family history of CAD, n (%) | 8 (15.1) | 18 (11.2) | 0.45 |
| Hypertension, n (%) | 37 (69.8) | 121 (75.2) | 0.44 |
| Dyslipidemia, n (%) | 42 (79.2) | 137 (85.1) | 0.32 |
| Diabetes mellitus, n (%) | 32 (60.4) | 65 (40.4) | 0.009 |
| Prior CAD, n (%) | 23 (43.4) | 56 (34.8) | 0.26 |
| Statin use, n (%) | 39 (73.6) | 128 (79.5) | 0.37 |
| Acute coronary syndrome, n (%) | 9 (17.0) | 27 (16.8) | 0.97 |
| Multi-vessel disease, n (%) | 23 (43.4) | 55 (34.2) | 0.23 |

| | | | |
|---|---|---|---|
| Triglyceride (mg/dL) | 136.0 (93.5–168.5) | 119.0 (88.3–168.0) | 0.25 |
| Total cholesterol (mg/dL) | 163.7 ± 40.0 | 169.1 ± 44.3 | 0.43 |
| HDL cholesterol (mg/dL) | 42.7 ± 9.6 | 47.6 ± 12.5 | 0.02 |
| LDL cholesterol (mg/dL) | 96.3 ± 28.9 | 100.8 ± 35.8 | 0.41 |
| HbA1c (NGSP) (%) | 6.63 ± 1.16 | 6.30 ± 0.93 | 0.03 |
| eGFR (ml/min/1.73m$^2$) | 62.3 ± 14.3 | 63.4 ± 13.8 | 0.62 |
| High-sensitivity CRP (mg/dL) | 0.10 (0.03–0.28) | 0.07 (0.03–0.14) | 0.37 |
| BNP (pg/mL) | 30.4 (16.6–71.6) | 42.0 (19.8–91.6) | 0.17 |
| KetoA (pg/mL) | 244.6 (149.2–315.0) | 291.6 (203.9–393.1) | 0.03 |
| KetoB (pg/mL) | 720.5 (551.6–876.5) | 818.4 (641.1–1103.6) | 0.01 |
| HYA + HYC (pg/mL) | 241.7 (142.0–792.5) | 312.5 (176.6–681.8) | 0.40 |
| HYB (pg/mL) | 557.1 (388.4–828.2) | 624.8 (405.9–893.5) | 0.31 |
| Lipid metabolite levels at follow-up | | | |
| KetoA (pg/mL) | 281.5 (154.5–444.2) | 272.1 (179.0–414.0) | 0.94 |
| KetoB (pg/mL) | 727.1 (473.1–917.0) | 790.4 (533.9–1095.1) | 0.11 |

| | | | |
|---|---|---|---|
| HYA + HYC (pg/mL) | 379.8 (191.3–827.4) | 290.3 (176.1–675.9) | 0.30 |
| HYB (pg/mL) | 617.3 (408.8–990.6) | 621.6 (432.2–886.6) | 0.89 |
| Change value of lipid metabolite levels during follow-up | | | |
| KetoA (pg/mL) | 39.0 (-94.9–188.3) | 15.2 (-174.5–154.5) | 0.27 |
| KetoB (pg/mL) | 10.3 (-283.6–345.3) | -36.6 (-397.5–330.6) | 0.49 |
| HYA + HYC (pg/mL) | 81.1 (-146.7–280.6) | -17.6 (-244.2–131.2) | 0.06 |
| HYB (pg/mL) | 70.8 (-204.8–333.9) | -4.65 (-343.6–254.6) | 0.19 |
| Abbreviations: BNP, brain natriuretic peptide; CAD, coronary artery disease; CRP, C-reactive protein; CUC, cholesterol up-take capacity; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high density lipoprotein; LDL, low density lipoprotein cholesterol. | | | |

**Table2. Uni- and multivariate logistic regression analysis for revascularization**

| Variables | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | p-value | OR | 95% CI | p-value |
| Age | 0.99 | 0.96–1.02 | 0.37 | | | |
| Male sex | 2.26 | 0.89–5.70 | 0.09 | | | |
| Presence of diabetes mellitus | 2.25 | 1.19–4.24 | 0.01 | 2.05 | 1.05–3.98 | 0.04 |
| Prior CAD | 1.44 | 0.76–2.71 | 0.26 | | | |
| Multi-vessel disease | 1.48 | 0.78–2.78 | 0.23 | | | |
| Triglyceride level | 1.00 | 1.00–1.00 | 0.17 | | | |
| HDL cholesterol level | 0.96 | 0.93–0.99 | 0.01 | 0.97 | 0.94–1.00 | 0.048 |
| LDL cholesterol level | 1.00 | 0.99–1.00 | 0.41 | | | |
| KetoA level (per 100 pg/mL increase) | 0.87 | 071–1.07 | 0.17 | | | |
| KetoB level (per 100 pg/mL increase) | 0.90 | 0.83–0.98 | 0.02 | 0.90 | 0.82–0.98 | 0.01 |
| Abbreviations: CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OR, odds ratio. | | | | | | |

**Table 3. Laboratory data at baseline and follow-up, and mean change value during follow-up in all patients**

| | Baseline | Follow-up | Mean change value | 95% CI | | Standard Error | t-value | p-value |
|---|---|---|---|---|---|---|---|---|
| | | | | Lower | Upper | | | |
| KetoA (pg/mL) | 277.2 (190.8–380.5) | 273.0 (170.3–415.6) | 30.6 | -16.0 | 77.3 | 23.7 | 1.30 | 0.20 |
| KetoB (pg/mL) | 780.7 (604.3–1026.6) | 769.1 (518.3–1075.5) | -28.8 | -131.6 | 73.9 | 52.1 | -0.55 | 0.58 |
| HYA + HYC (pg/mL) | 299.6 (169.1–703.7) | 299.7 (176.4–713.1) | 95.6 | -169.1 | 360.2 | 134.3 | 0.71 | 0.48 |
| HYB (pg/mL) | 597.4 (403.2–879.4) | 619.6 (417.8–940.2) | 62.8 | -117.6 | 243.2 | 91.5 | 0.69 | 0.49 |
| Triglyceride (mg/dL) | 121.0 (89.0−168.0) | 108.0 (79.8−151.3) | -17.6 | -26.9 | -8.3 | 4.73 | -3.72 | <0.001 |
| Total cholesterol (mg/dL) | 167.8 ± 3.0 | 142.8 ± 2.3 | -24.9 | -30.5 | -19.3 | 2.85 | -8.75 | <0.001 |
| HDL cholesterol (mg/dL) | 46.2 ± 0.81 | 46.9 ± 0.83 | 0.68 | -0.39 | 1.76 | 0.55 | 1.26 | 0.21 |
| LDL cholesterol (mg/dL) | 99.7 ± 2.3 | 76.7 ± 1.7 | -22.8 | -27.6 | -18.0 | 2.43 | -9.38 | <0.001 |
| HbA1c (NGSP) (%) | 6.38 ± 0.07 | 6.41 ± 0.06 | 0.03 | -0.04 | 0.11 | 0.04 | 0.86 | 0.39 |
| HbA1c (NGSP) (%), in patients with DM | 7.09 ± 0.11 | 7.03 ± 0.10 | -0.05 | -0.21 | 0.10 | 0.07 | -0.70 | 0.49 |
| Abbreviations: DM, diabetes mellitus, HbA1c, hemoglobin A1c; HDL, high density lipoprotein; LDL, low density lipoprotein cholesterol. | | | | | | | | |

**Figure title and legends**

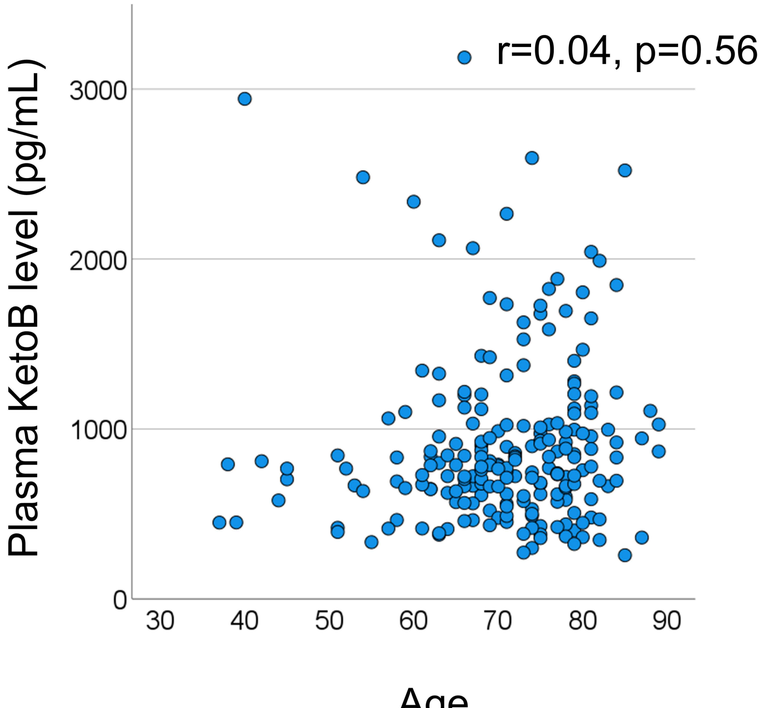
**Figure 1. Relationship between plasma KetoB levels and patients' characteristics**

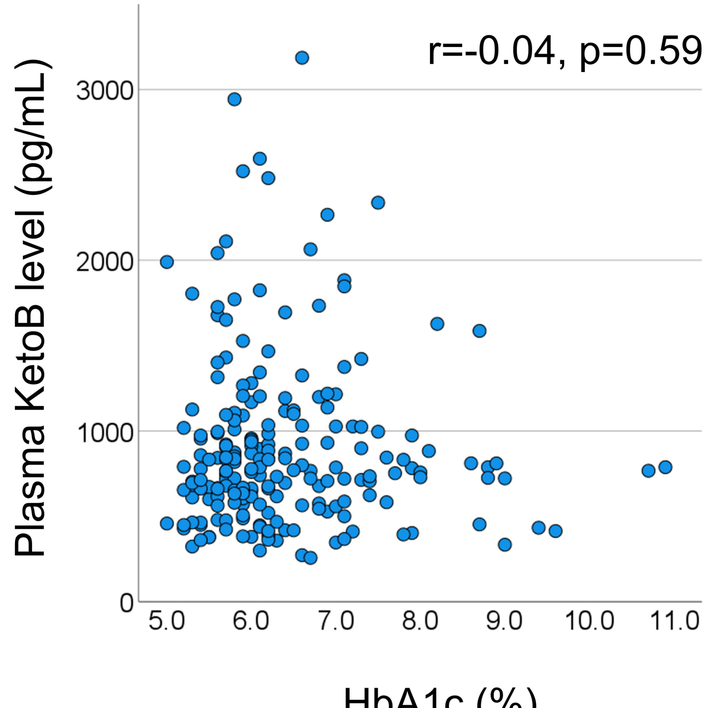
BMI, body mass index; HbA1c, hemoglobin A1c; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein

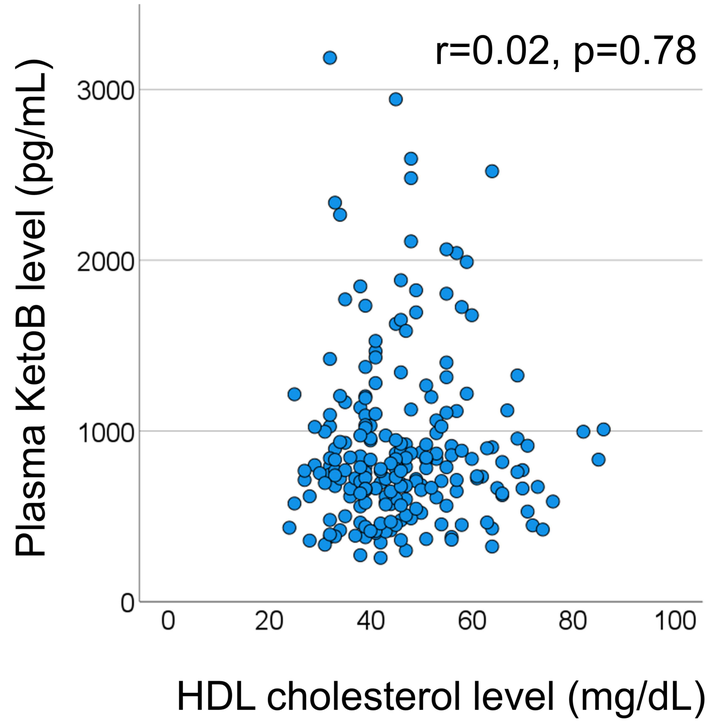
**Figure 2. mRNA expression in macrophages and neutrophils by adding HYA, KetoA, and KetoB**

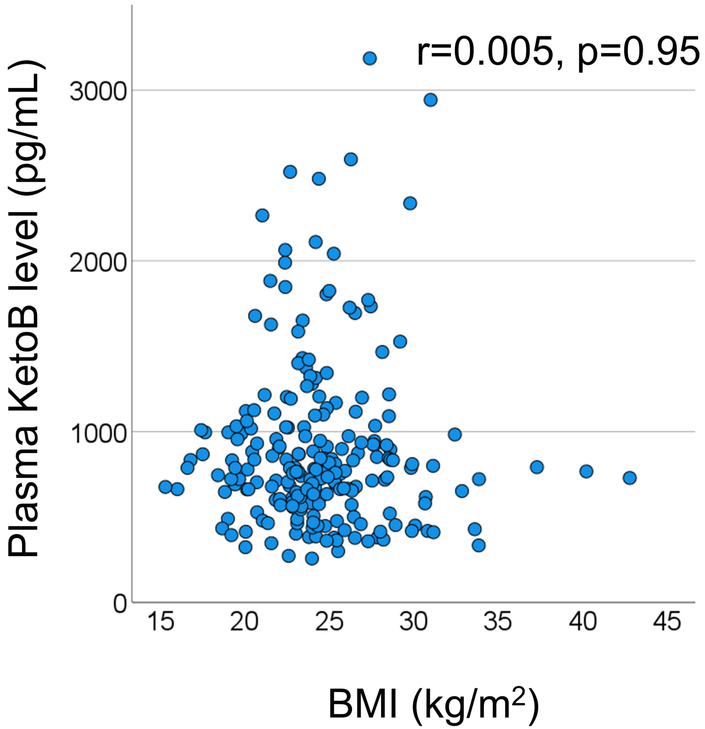
(A–D)： RAW 264.7 was incubated with Kdo2 for 1 hour, followed by the addition of 10 μM of HYA, KetoA, and KetoB for 1 hour at 37˚C. IL1-β (A), IL-6 (B), TNF-a (C), and CCL-2 (D) mRNA were analyzed with real-time PCR. Data are expressed as fold change from the vehicle, mean ± S.E. (n=4). *, **, ***, **** indicates $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$.

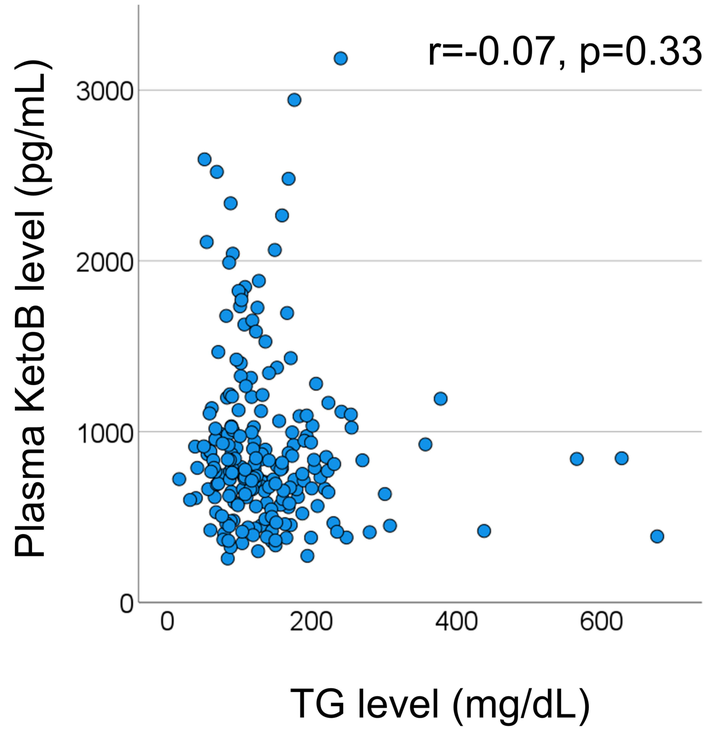
(E–H): HL-60 was incubated with Kdo2 for 1 hour, followed by adding 10 μM of HYA, KetoA, and KetoB for 2 hours at 37℃. IL1-β (E), IL-6 (F), TNF-a (G), and CCL-2 (H) mRNA were analyzed with real-time PCR. Data are expressed as fold change from the vehicle, mean ± S.E. (n=5). * Indicates $p < 0.05$.

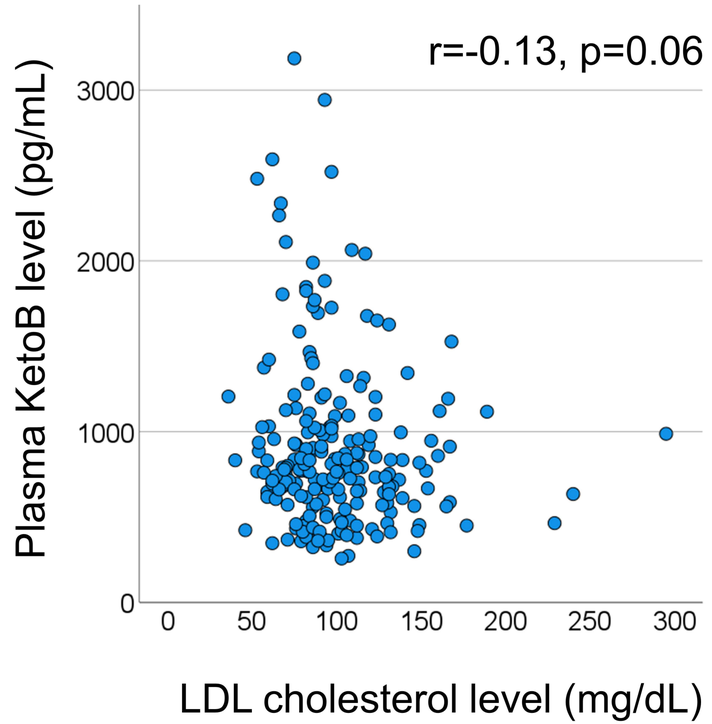

r=0.04, p=0.56
Plasma KetoB level (pg/mL)
3000
2000
1000
0
30
40
50
60
70
80
90
Age


r=-0.04, p=0.59
Plasma KetoB level (pg/mL)
3000
2000
1000
0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
HbA1c (%)


r=0.02, p=0.78
Plasma KetoB level (pg/mL)
3000
2000
1000
0
0
20
40
60
80
100
HDL cholesterol level (mg/dL)


r=0.005, p=0.95
Plasma KetoB level (pg/mL)
3000
2000
1000
0
15
20
25
30
35
40
45
BMI (kg/m2)


r=-0.07, p=0.33
Plasma KetoB level (pg/mL)
3000
2000
1000
0
0
200
400
600
TG level (mg/dL)


r=-0.13, p=0.06
Plasma KetoB level (pg/mL)
3000
2000
1000
0
0
50
100
150
200
250
300
LDL cholesterol level (mg/dL)

## Macrophages

## Neutrophils

A

IL-1β

mRNA expression (AU)

1.1
1.0
0.9
0.8
0.7
0.6

*
**

Veh HYA KetoA KetoB

+Lipid A

B

IL-6

mRNA expression (AU)

1.5
1.0
0.5
0.0

****
***
**

Veh HYA KetoA KetoB

+Lipid A

E

IL-1β

mRNA expression (AU)

1.5
1.0
0.5
0.0

*
*

Veh HYA KetoA KetoB

+Lipid A

F

IL-6

mRNA expression (AU)

1.5
1.0
0.5
0.0

Veh HYA KetoA KetoB

+Lipid A

C

TNF-α

mRNA expression (AU)

1.5
1.0
0.5
0.0

Veh HYA KetoA KetoB

+Lipid A

D

CCL-2

mRNA expression (AU)

1.5
1.0
0.5
0.0

Veh HYA KetoA KetoB

+Lipid A

G

TNF-α

mRNA expression (AU)

1.5
1.0
0.5
0.0

Veh HYA KetoA KetoB

+Lipid A

H

CCL-2

mRNA expression (AU)

1.5
1.0
0.5
0.0

Veh HYA KetoA KetoB

+Lipid A

## Methods

214 retrospective patients

Index PCI

Clinical follow-up
(≥ 3months)

Revascularization
(n=53)

CAG without
revascularization
(n=161)

## Linoleic acid metabolic pathway of gut microbiome

Linoleic acid

HYA

KetoA

KetoC

HYC

KetoB

Conjugated
linoleic acid

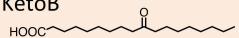
HYB

Oleic acid

## Summary

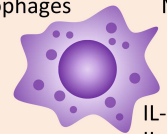
- Decreased plasma KetoB levels were independently associated with subsequent revascularization after PCI
- KetoB contributed to anti-inflammation

KetoB

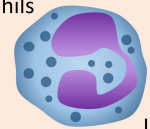



Macrophages

Neutrophils



IL-1β ↓
IL-6 ↓



IL-1β ↓