I. Introduction

Coronary artery disease (CAD) is a common cause of death in Japan, causing about 200,000 deaths each year\(^1\). The use of percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) have reportedly improved patient outcomes since their introduction\(^2\). Thus, early detection and treatment for CAD may further reduce the mortality rate. Asymptomatic CAD occurs in 15% of patients aged <70 years and in 28% of those aged >70 years\(^3\). Therefore, a highly sensitive screening method is required to detect CAD in these individuals. Some studies report that stress testing with electrocardiography, echocardiography, and scintigraphy monitoring, and the use of coronary computed tomography (CT) to measure the coronary calcium level are useful for CAD screening\(^4-8\). However, these tests are expensive and time-consuming in some clinical settings, which reduces their feasibility.

Repeated myocardial ischemia-reperfusion (MIR) occurs in patients with CAD, wherein intermittent blood flow restriction is followed by re-canalization\(^9\). Reperfusion and reoxygenation after myocardial ischemia cause the release of a large number of reactive oxygen species (ROS)\(^10\). We have focused on the oxidative stress that occurs in MIR. Serum bilirubin is part of the biological defense system that is activated in response to the presence of ROS\(^11\). After reoxygenation, ROS rapidly oxidize bilirubin to form u-biopyrrin, which is detectable in the urine. This study investigates the urinary-biopyrrin (u-biopyrrin) levels of CAD patients to assess their utility as a parameter for CAD assessment.

Materials and Methods: We retrospectively analyzed 32 CAD patients, 11 non-CAD patients, and 5 post-revascularization patients (PRP). The 32 CAD patients were further divided into subgroups based on the number of vessels involved, namely, 3- or 2-vessel disease (VD) (n=25 and 7, respectively) and the type of myocardial ischemia symptom, namely, unstable angina pectoris (UAP), effort angina pectoris (EAP), or silent myocardial ischemia (SMI) (n=9, 14, and 9, respectively). Participant data were compared between each group. To compare the association between the severity of CAD and u-biopyrrin value, the severity of CAD was evaluated using SYNTAX I and SYNTAX II scores. Results: The u-biopyrrin levels were higher in the CAD group than in the non-CAD and PRP groups. There was no significant difference in u-biopyrrin value between the 3-VD and 2-VD patients, or among UAP, EAP, and SMI patients. The anatomical and clinical severity scores indicated by SYNTAX I and II scores were moderately correlated with u-biopyrrin levels and treatment risk in the receiver operating characteristic curve analysis. The u-biopyrrin level showed a cut-off value of 2.1 mmol/g.cre (AUC, 0.739; sensitivity, 43.75%; specificity, 93.75%). Conclusions: The u-biopyrrin levels were greater in CAD patients than in non-CAD and PRP patients. U-biopyrrin levels moderately correlated with the anatomical and clinical severity of CAD.
patients were enrolled in the study. Patients who planned to undergo CABG or PCI for CAD were included in the CAD group (n=32). Urinary biopurrin were assessed before revascularization therapy. Participants with intact coronary arteries were included in the non-CAD group (n=11). Of the 11 non-CAD patients who underwent coronary angiography (CAG), 6 underwent the CAG assessment prior to non-cardiac surgery. Five patients had atypical chest oppression and non-specific ST-T changes on electrocardiography. The PRP group included five patients who previously underwent CABG and PCI. The graft and coronary artery patency were confirmed using intraoperative near-infrared fluorescence angiography and X-ray CAG in the five PRPs. We used the CAG results of PRPs performed in the postoperative period after revascularization (mean, 11.8 ± 3.8 months after revascularization therapy). Urine sample were collected at least 48 hours after CAG in all participants. Patients who underwent hemodialysis for chronic kidney disease (CKD) were excluded from the study. Patients taking steroids with antioxidant properties were also excluded because steroids reduce ROS production.

Furthermore, the CAD group was divided into subgroups according to the anatomical severity (number of lesions) and the clinical symptom. Anatomical classification was based on CAG. The CAD group (n=32) was sub-classified into the triple-vessel disease (3-VD) (n=25) and double-vessel disease (2-VD) groups (n=7). The CAD patients were also classified into subgroups based on their myocardial ischemia symptoms as follows: unstable angina pectoris (UAP) group (n=9), effort angina pectoris (EAP) group (n=14), and silent myocardial ischemia (SMI) group (n=9). UAP was defined as typical chest pain or other ischemic symptoms occurring at rest or with minimal exertion. The UAP group included patients with symptoms exceeding those of grade II according to the Canadian Cardiovascular Society classification guidelines. EAP was defined as chest pain or discomfort most often occurring with activity or stress. SMI is defined as objective documentation of myocardial ischemia in the absence of angina or anginal equivalents. In this study, patients who underwent CAG due to abnormal electrocardiogram (ECG) findings (5 patients) or reduced cardiac function (3 patients) and those who underwent preoperative CAG for non-cardiac surgery without chest pain (1 patient) were included in SMI group.

To compare the association between the anatomical severity and u-biopurrin value, the anatomical severity was evaluated using SYNTAX I scores in CAD group. Additionally, clinical severity of CAD was indicated using the SYNTAX II score, which combined the following variables: age, creatinine clearance, ejection fraction, sex, chronic obstructive pulmonary disease (COPD), and peripheral vessel disease. When calculating the SYNTAX I and II scores, the estimated glomerular filtration rate
(eGFR) was used instead of the creatinine clearance value. The SYNTAX II score was also calculated for the PCI and CABG scores.

Data collection and measurement

Patient data regarding symptoms and past medical history were collected from interviews and medical charts. All participants underwent urine collection and resting u-biopyrrin measurements for at least 48 hours after CAG. Spot urine samples (5 mL) were assessed after collection. The urine samples were centrifuged at 3,000 rpm and u-biopyrrin levels were measured by performing enzyme-linked immunosorbent assay using antibody 24G7 (Biopyrrin EIA Kit; Shino-Test, Tokyo, Japan).22) The total bilirubin concentration was measured using Vanadate Oxidation Total Bilirubin E-HR with a detection limit of 0.2 mg/dL (Wako Pure Chemical Industries, Osaka, Japan). The creatinine concentration in urine is also measured using an enzymatic assay, and the u-biopyrrin is expressed as a value adjusted by the creatinine concentration.23, 24) An auto analyzer (Beckman Coulter, Fullerton, USA) was used to measure serum levels of total bilirubin, creatinine, blood urea nitrogen (BUN), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), total cholesterol, and B-type natriuretic peptide (BNP).

Definitions

COPD was defined as having a forced expiratory volume of <700 mL in 1 second and/or emphysematous changes. Patients were considered to have CKD if the eGFR was <45 mL/min/1.73 m².

Ethical considerations

The study protocol was designed and performed in accordance with the guidelines of the Ethics Committee for Clinical Research at the Kochi Medical School (IRB approval number 27–91, approval date April 16 2016). We also followed the Helsinki Declaration Principles (version 2013) to conduct this study. Written informed consent was given by every participants.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Patient characteristics were analyzed using Pearson’s chi-square test with Bonferroni correction. Urine, blood test findings, and SYNTAX score were analyzed using the Kruskal-Wallis test. If significant differences were detected, the Mann-Whitney U test with Bonferroni correction for multiple comparisons was used. To obtain the optimal cut-off for u-biopyrrin levels, receiver operating characteristic (ROC) curve analysis was performed. The cut-off values were determined based on the minimum values of the square root of [(1-sensitivity)² + (1-specificity)²], which indicated the minimum distance from the points on the ROC curve. Values of p < 0.05 were considered statistically significant. The statistical analysis was performed using JMP 14 software (SAS Institute, Cary, NC, USA).

III. Results

Patient characteristics

In total, data from 48 participants were included in the analysis. Patient characteristics are summarized and shown in Table 1. The peripheral artery disease (PAD) incidence rate was significantly higher in CAD. The prevalence rates of risk factors, including diabetes mellitus, hypertension, dyslipidemia, current smoking, and comorbidities were also not significantly different between the groups. Serum levels of total-bilirubin, creatinine, BUN, LDH, CPK, and BNP.

There were no significant differences between the groups in terms of serum levels of total-bilirubin, creatinine, BUN, LDH,
CPK, and BNP (Table 1). Creatinine values were ≤ 1.8 mg/dL, and eGFR was >50 mL/min/1.73 m². Thus, the renal function had no effect on urinary excretion or level of u-biopyrins.

Urinary biopyrins data

The u-biopyrрин levels are reported for all groups (Fig. 3). The u-biopyrрин levels were higher in the CAD group than in the non-CAD and PRP groups (2.69±0.55, 0.49±0.22, 0.17±0.18 mmol/g.cre, respectively, CAD vs. non-CAD, p=0.004 and CAD vs. PRP, p=0.019). There was no significant difference in the u-biopyrрин levels between the non-CAD and PRP groups. Furthermore, the u-biopyrрин level tended to be higher in the 3-VD subgroup than in the 2-VD subgroup with no significant differ-

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| Clinical demographics | CAD (%) | non-CAD (%) | PRP (%) | p value |
|------------------------|---------|-------------|---------|---------|
| Number of patients     | 32      | 11          | 5       |         |
| Age (years)            | 70.6 ± 1.5 | 80.6 ± 2.1 | 70.6 ± 4.4 | 0.007*1 |
| Gender (female)        | 13      | 7           | 1       | 0.219   |

| Anatomical classification | 3-VD | 2-VD |
|---------------------------|------|------|
| 3-VD                      | 25 (78.1) |      |
| 2-VD                      | 7 (21.9)    |      |

| Symptomatic classification | UAP | EAP | SMI |
|---------------------------|-----|-----|-----|
| UAP                       | 9 (28.1) |   |    |
| EAP                       | 14 (43.8) | | |
| SMI                       | 9 (28.1)    | | |

| Cardiovascular risk factors | | |
|-----------------------------| | |
| Hypertension                | 30 (93.8) | 10 (90.9) | 5 (100) | 0.785 |
| Dyslipidemia                | 21 (65.6) | 7 (63.6) | 2 (40.4) | 0.544 |
| Diabetes                    | 13 (40.6) | 1 (9.1) | 2 (40.0) | 0.152 |
| PCI history                 | 6 (18.8) | 0 (0) | 2 (40.0) | 0.3 |
| CABG history                | 0 (0) | 0 (0) | 3 (60.0) | 0.001*2 |
| CVA                        | 8 (25.0) | 3 (27.3) | 4 (80.0) | 0.274 |
| CKD                        | 2 (6.3) | 1 (9.1) | 1 (20.0) | 0.583 |
| PAD                        | 13 (40.6) | 1 (9.1) | 0 (0) | 0.044*3 |
| Current smoking            | 14 (43.8) | 2 (18.2) | 1 (20.0) | 0.232 |
| COPD                       | 4 (12.5) | 0 (0) | 0 (0) | 0.336 |

| Blood test results | | |
|-------------------| | |
| T-Bilirubin (mg/dL) | 0.6 ± 0.1 | 0.7 ± 0.3 | 0.6 ± 0.1 | 0.574 |
| CPK (U/L)          | 147.7 ± 16.3 | 91.7 ± 15.2 | 127.4 ± 10.6 | 0.6 |
| LDH (U/L)          | 219.3 ± 16.2 | 195.5 ± 15.2 | 168.8 ± 10.6 | 0.511 |
| Creatinine (mg/dL) | 0.9 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.2 | 0.967 |
| BUN (mg/dL)        | 18.7 ± 1.5 | 18.1 ± 2.1 | 20.3 ± 3.4 | 0.922 |
| eGFR (mL/min/1.73m²) | 66.9 ± 4.0 | 53.2 ± 4.7 | 61.2 ± 7.6 | 0.221 |
| BNP (pg/mL)        | 159.7 ± 62.9 | 62.3 ± 18.4 | 88.6 ± 24.4 | 0.945 |

| Echocardiography | | |
|------------------| | |
| EF (%)           | 62.2 ± 2.1 | 65.5 ± 2.3 | 64.3 ± 3.8 | 0.859 |

CAD: coronary artery disease, non-CAD: non-coronary artery disease, PRP: post-reperfusion patient, 3-VD: 3-vessel disease, 2-VD: 2-vessel disease, UAP: unstable angina pectoris, EAP: effort angina pectoris, SMI: silent myocardial ischemia, PCI: percutaneous coronary intervention, CABG: coronary artery bypass grafting, CVA: cerebral vascular accident, CKD: chronic kidney disease, PAD: peripheral artery disease, COPD: chronic obstructive pulmonary disease, T-Bilirubin: Total-Bilirubin, CPK: creatinine phosphokinase, LDH: lactate dehydrogenase, BUN: blood urea nitrogen, eGFR: estimated glomerular filtration rate, BNP: B-type natriuretic peptide, EF: ejection fraction.

The patient characteristics were analyzed using Pearson’s chi-square test adjusted by the Bonferroni method. *1The value was significantly higher in the non-CAD group than in the CAD and PRP groups (p=0.002 and 0.039, respectively). *2The value was significantly higher in the PRP group than in the CAD and non-CAD groups (both p<0.001). *3The value was significantly higher in patients with CAD than in the non-CAD and PRP groups (p=0.001 and 0.018, respectively).
ence (2.99±0.67 vs. 1.58±0.64 mmol/g.cre, p=0.293). The u-biopyrrins level was 3.19±1.05, 2.01±0.92, and 3.11±1.08 mmol/g.cre in the UAP, EAP, and SMI subgroups, respectively, and was not significantly different between groups (UAP vs. EAP, p=0.395; EAP vs. SMI, p=0.441; and UAP vs. SMI, p=0.926).

Association between anatomical and symptomatic severity with biopyrrins level

The mean value of SYNTAX I scores was 22.4±1.65 in the CAD group, with 62.5% of the CAD group in the low (0–22) SYNTAX I score quantile, 25.0% in the intermediate (23–32) quantile, and 12.5% in the high (>32) quantile. For anatomical classification, the mean SYNTAX I score was 23.5±1.98 in the 3-VD group and 18.6±2.18 in the 2-VD group. For symptomatic classification, the mean SYNTAX I score was 25.1±5.42 in the UAP group, 18.2±2.87 in the EAP group, and 25.5±3.76 in the SMI group. The correlation diagram comparing the anatomical severity score (derived from the results of SYNTAX I score of the CAD group) and the u-biopyrrins level showed a moderate correlation (R²=0.36) (Fig. 4A).

Risk prediction was calculated using SYNTAX II scores for patients who underwent PCI and CABG. The mean values were 38.2±1.75 and 34.3±2.91 in the PCI and CABG, respectively (Table 2). The mean SYNTAX II score values for the risk of PCI were 37.9±1.79 and 25.2±3.57 in the 3-VD and 2-VD subgroups, respectively. For the risk of CABG, the mean values were 34.2±3.37 and 36.8±2.59 in the 3-VD and 2-VD subgroups, respectively, with no significant difference. The SYNTAX II score mean values for the risk of PCI were 39.4±3.91, 36.2±2.87, and 39.6±3.76 in the UAP, EAP, and SMI subgroups, respectively. Risk values for CABG were 33.6±1.80, 31.5±2.39, and 30.8±2.51 in the UAP, EAP, and SMI subgroups, respectively. The correlation diagram comparing the risk prediction (derived from the results of the SYNTAX II score of the CAD group) and the u-biopyrrin levels showed a moderate correlation (R²=0.28 and R²=0.17 for PCI, and CABG, respectively) (Fig. 4B and C).

According to the ROC curve analysis, the optimal cut-off value of u-biopyrrin levels was 2.1 mmol/g.cre (AUC = 0.739, p = 0.062 ; Fig. 5). All patients who had a u-biopyrrin level > 2.1 mmol/g.cre were included in the CAD group. Furthermore, the u-biopyrrins value was more than the cut-off value in 43.8% of patients in the CAD group. In contrast, 100% of patients in the non-CAD group had u-biopyrrin levels less than the cut-off value. Therefore, the cut-off for the u-biopyrrins value

Fig. 3  Levels of urinary biopyrin (u-biopyrin).
Among CAD, non-CAD, and PRP groups, the u-biopyrin level is significantly higher in the CAD group. The 2-VD and 3-VD groups show no significant differences. U-biopyrin levels are not significantly different between the UAP, EAP, and SMI groups.

CAD: coronary artery disease, PRP: post-revascularization patient, 2-VD: 2 vessel disease, 3-VD: 3 vessel disease, UAP: unstable angina pectoris, EAP: effort angina pectoris, SMI: silent angina pectoris.
*indicates significance.

Fig. 4  Correlation diagram comparing urinary biopyrin (u-biopyrin) and severity of coronary artery disease (CAD).
(A) Correlation diagram comparing u-biopyrin levels and SYNTAX I scores. A moderate correlation with the correlation function, R²=0.38. (B, C) Correlation diagram comparing the u-biopyrin levels and SYNTAX II scores shows risk for PCI and CABG results in a moderate correlation with the correlation function, R²=0.28 and 0.17 for PCI and CABG, respectively.
PCI: percutaneous coronary intervention, CABG: coronary artery bypass grafting.
*indicates significance.
was 2.1 mmol/g.cre with 43.8% sensitivity, 93.8% specificity, 93.3% positive predictive value, and 45.5% negative predictive value. According to anatomical classification, 48.0% and 40.0% of patients had more than the cut-off value in 3-VD and 2-VD groups, respectively (Fig. 6B), and according to symptomatic severity, 66.7%, 21.4%, and 66.7% of patients had more than the cut-off value in the UAP, EAP, and SMI groups, respectively (Fig. 6C).

### IV. Discussion

The u-biopyrrin levels were higher in the CAD patients than in the non-CAD and PRP patients. This study analyzed the differences in u-biopyrrin levels between participants with and without CAD to assess the feasibility of using u-biopyrrin levels as a parameter for CAD. Furthermore, the SYNTAX I scores were moderately correlated to the u-biopyrrin levels. We thought that u-biopyrrin levels reflected ROS production and u-biopyrrin production depended on the volume of the myocardium which was exposed to repeated ischemia and reperfusion. Further, SYNTAX II scores were moderately correlated to the u-biopyrrin levels. And urinals analysis revealed a tendency for higher u-biopyrrin in UAP patients than in EAP patients. It is thought that the incidence of myocardial ischemia and reperfusion associated with UAP is more frequent than with EAP. These results support the hypothesis that myocardial volume and repetition of ischemia reperfusion might increase biopyrrin production, and that u-biopyrrin levels may be a viable marker for CAD assessment. Additionally, the present study revealed a tendency towards increased u-biopyrrin levels in patients with SMI without
chest pain or chest oppression, although with no statistical difference. This detection might have an advantage in screening for SMI for asymptomatic individuals to prevent fatal cardiac events including sudden death. We also thought that u-biopyrrin level may reflect the anatomical or clinical CAD severity. Furthermore, we also reported here the cut-off value of u-biopyrrin for suspected angina pectoris. The u-biopyrrin assessment for which the cut-off value was 2.1 mmol/g.c.re had 93.8% specificity and 93.3% positive predictive value. We discussed the false positives and false negatives of the u-biopyrrin test for CAD. U-biopyrrin value was below the cut-off value in all participants in the non-CAD group (see Fig. 6A). In total, 66.7% of the patients with UAP, and 66.7% of the patients with SMI showed positive u-biopyrrin (see Fig. 6C). Thus, these results might show the high specificity of urinalysis for biopyrins. Therefore, this might be a preliminary study showing that biopirin measurement may be effective for detecting angina pectoris, although further research is required.

Screening tests using urinalysis are time and cost-efficient tools compared to the other methods for detected CAD\textsuperscript{25, 26}. In urinalysis, sample collection is easy and the evaluation method for detecting CAD is not particularly complicated. The u-biopyrrin level increases immediately after ischemia-reperfusion and remains elevated for 48 hours\textsuperscript{27}. Because of this property, u-biopyrrin levels may be a suitable screening marker for CAD. We believe that a future prospective cohort study will be required to confirm our findings.

Even though the present study showed 93.8% specificity for the CAD test using the u-biopyrrin levels, a low-frequency ischemic attack might not increase ROS and biopyrrin levels\textsuperscript{28}. It is conceivable that this could result in a false negative. Therefore, it is important to consider that assessment of u-biopyrrin levels may result in some false negatives, therefore, u-biopyrrins test is not recommended for individuals who have angina-like symptoms such as chest pain or ECG abnormalities. They should undergo enhanced coronary-CT or x-ray coronary angiography for evaluation of the coronary arteries. On the other hand, there was an increase in the levels of u-biopyrrin in CAD patients that showed high specificity and positive predictive values after ROC curve analysis, although there might be a risk for false positives in CAD screening using u-biopyrrin assessment. It is possible, for example, that other diseases associated with high levels of oxidative stress also result in high u-biopyrrin levels\textsuperscript{29, 30}.

Atherosclerotic diseases are often caused by diabetes, dyslipidemia, and hypertension, which may cause oxidative stress. ROS and reactive nitrogen species (RNS) activities are reportedly increased in hypertensive patients, resulting in vascular damage\textsuperscript{31, 32}. In diabetes mellitus, increased oxidative stress due to increased nicotinamide adenine dinucleotide phosphate oxidase activity and polyol metabolism reportedly result in vascular endothelial cell damage\textsuperscript{33}. Myocardial injury due to increased xanthine oxidase activity and ROS has been reported in patients with dyslipidemia\textsuperscript{34}. Although there was no significant difference in the complication rates of these diseases, it will be necessary to study diseases that affect u-biopyrrin production in future. Although u-biopyrrin level was affected by creatinine concentration\textsuperscript{35, 36}, the influence of renal function on biopyrrin excretion could not be examined in this study, and there are no similar previous reports. Elucidating the influence of renal function and diseases that influence oxidative stress production will require future studies.

Study Limitations

This study had several limitations. First, we assessed the u-biopyrrin levels in only a small sample of participants; therefore, the statistical analysis might be insufficiently powered. However, we believe that CAD is associated with increased u-biopyrrin levels. Furthermore, we also reported here the cut-off value of u-biopyrrin level for suspected angina pectoris. Therefore, we believe that a prospective cohort study is required in the future. Second, the study included patients with CAD before undergoing revascularization therapy such as CABG or PCI. Because of the large number of patients in the surgery department included in this study, there were many cases of 3-VD and 2-VD but no cases of IVD, which might introduce the possibility of bias.

V. Conclusion

The u-biopyrrin levels were greater in CAD patients than in non-CAD and PRP patients. U-biopyrrin levels moderately correlated with the anatomical and clinical severity of CAD. This study showed that u-biopyrrin may be useful as a CAD screening parameter.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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