Clinical Chemistry 59:9
1330–1337 (2013)

Processed B-Type Natriuretic Peptide Is a Biomarker of Postinterventional Restenosis in Ischemic Heart Disease

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BACKGROUND: Restenosis, a condition in which the lesion vessel renarrows after a coronary intervention procedure, remains a limitation in management. A surrogate biomarker for risk stratification of restenosis would be welcome. B-type natriuretic peptide (BNP) is secreted in response to pathologic stress from the heart. Its use as a biomarker of heart failure is well known; however, its diagnostic potential in ischemic heart disease is less explored. Recently, it has been reported that processed forms of BNP exist in the circulation. We hypothesized that circulating processed forms of BNP might be a biomarker of ischemic heart disease.

METHODS: We characterized processed forms of BNP by a newly developed mass spectrometry–based detection method combined with immunocapture using commercial anti-BNP antibodies.

RESULTS: Measurements of processed forms of BNP by this assay were found to be strongly associated with presence of restenosis. Reduced concentrations of the amino-terminal processed peptide BNP(5–32) relative to BNP(3–32) [as the index parameter BNP(5–32)/BNP(3–32) ratio] were seen in patients with restenosis [median (interquartile range) 1.19 (1.11–1.34), n = 22] vs without restenosis [1.43 (1.22–1.61), n = 83; $P < 0.001$] in a cross-sectional study of 105 patients undergoing follow-up coronary angiography. A sensitivity of 100% to rule out the presence of restenosis was attained at a ratio of 1.52.

CONCLUSIONS: Processed forms of BNP may serve as viable potential biomarkers to rule out restenosis.

Percutaneous coronary intervention (PCI)[4] procedures are widely used today to treat coronary artery disease *(1, 2)*. Even with use of drug-eluting stents, restenosis (as defined as renarrowing of the treated lesion at approximately 3–6 months after the procedure, which often requires another intervention procedure to treat) still remains a limitation and occurs in >10% of patients. The pathology underlying restenosis is complex, involving a multitude of processes (inflammatory response to endothelial denudation and subintimal hemorrhage triggered by angioplasty followed by vascular smooth muscle cell proliferation and migration, extracellular matrix formation, and vascular remodeling) *(3)*. The mechanisms of restenosis are not yet fully understood, and, therefore, targeted medical intervention and biomarkers reflective of the process have yet to be developed to improve management of the condition and risk stratification. Clinical algorithms for the identification of patients at risk for this condition have not proven reliable, making clinical assessment of the condition difficult *(4–6)*. Owing to a compliant medical care system, patients undergoing an intervention procedure in Japan are generally given a follow-up angiogram at approximately 6 months to examine for presence of restenosis, but in most countries a follow-up angiogram is still limited to symptomatic patients. A surrogate biomarker that could help identify patients at risk for restenosis would therefore be welcome.

B-type natriuretic peptide (BNP) is a bioactive peptide that counteracts hemodynamic stress induced by various pathologic conditions through actions such as natriuresis and vasodilation *(7, 8)*. BNP is released into the circulation in large amounts during heart failure, allowing its measured circulating concentrations to be used in diagnosis of this condition *(7–10)*. BNP concentrations are also moderately increased in ischemic heart disease, but their diagnostic potential in this condition is less well explored *(11, 12)*. BNP is synthe-

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Received January 16, 2013; accepted April 22, 2013.
Previously published online at DOI: 10.1373/clinchem.2013.203406

[4] Nonstandard abbreviations: PCI, percutaneous coronary intervention; BNP, B-type natriuretic peptide; Aβ, amyloid β; DPP-IV, dipeptidyl-peptidase IV; CAG, coronary angiography; MS-IA, mass spectrometry–based immunoassay; IQR, interquartile range; CRP, C-reactive protein; OR, odds ratio.

sized as a propeptide, preproBNP(1–134), that undergoes rapid removal of a 26–amino acid (26-aa) signal peptide, resulting in the formation of a 108-aa prohormone, proBNP(1–108). Subsequently, proBNP(1–108) is cleaved by proteolytic enzymes furin and corin to release 2 processed peptides, the biologically inert 76-aa amino-terminal portion NT-proBNP(1–76) and the biologically active 32-aa molecule BNP(1–32) [see *(13)* for review]. Recently, other processed (proteolytic) forms of BNP [e.g., BNP(3–32), BNP(4–32), and BNP(5–32)] have been shown to exist in the circulation, but the clinical implications of these BNP peptides remain poorly understood *(14–16)*.

Protein processing via proteases is central to the metabolism of many peptides. In the heart, myofilament proteins such as troponin have been shown to be processed under ischemic conditions, which may lead to myocardial contractile dysfunction through effects on calcium-dependent muscle contraction responses *(17)*. Measurement of processed troponin peptides released into the circulation from damaged and/or necrotic cardiomyocytes has been suggested to be of potential use in risk stratification of patients with coronary syndromes *(18)*. There are other clinical situations in which processed proteins/peptides serve as diagnostic biomarkers, such as the use of amyloid β (Aβ) peptides in Alzheimer disease. The Aβ peptides generated through sequential proteolytic processing of the amyloid precursor protein by 2 enzymes, β-secretase and γ-secretase, have been shown to be reflective of Alzheimer disease pathophysiology [see *(19)* for review], with lower concentrations of Aβ42 (as a ratio to Aβ40) being associated with cognitive decline *(20)*. Protein processing is also the target of therapeutic interventions such as use of dipeptidyl-peptidase IV (DPP-IV) inhibitors, which inhibit protease processing of glucagon-like peptide 1 and glucose-dependent insulinotropic peptide in treatment of diabetes *(21–23)*. In the present study, we hypothesized that processing of BNP might have value as a diagnostic biomarker for ischemic heart disease and found that it is associated with restenosis.

Methods

PATIENTS AND PROTOCOLS

Between June 2007 and November 2011, we examined a total of 105 consecutive consenting patients with mildly increased BNP concentrations who underwent PCI with follow-up coronary angiography (CAG) approximately 6 months after the procedure. Patients were excluded if they had acute myocardial infarction, unstable angina pectoris, congestive heart failure, or chronic renal failure [serum creatinine >2.0 mg/dL (>176.8 μmol/L)], because of confounding effects on BNP concentrations. Patients with BNP concentrations >200 pg/mL were excluded because of possible confounding heart failure and other heart disease as described. Coronary angiograms were assessed by 2 experienced angiographers who were unaware of the results of analysis of BNP forms as described herein. Significant stenosis was defined as >50% narrowing of the coronary artery as determined by quantitative coronary angiography according to American Heart Association guidelines *(24)*.

Blood samples were obtained at time of follow-up CAG after PCI. Samples were transferred immediately into tubes containing EDTA-2Na and aprotinin (Neotube NP-EA0305, Nipro Corp.) and kept at 4 °C until plasma was separated by centrifugation within 6 h, and then stored at −80 °C until analysis. We measured plasma total BNP concentrations using a conventional enzyme immunoassay (Rapidpia, Sekisui Medical) *(25)*.

Nonstenotic concentrations of BNP(5–32)/BNP(3–32) ratio and BNP in this study were measured using blood samples from consenting patients diagnosed to not have coronary stenosis on diagnostic CAG (n = 66).

This study was approved by the ethics committee of the Graduate School of Medicine, the University of Tokyo, and written informed consent was obtained from each patient.

DETECTION OF BNP FORMS

We developed a mass spectrometry–based immunoassay (MS-IA) procedure (as described in detail in Supplemental Text, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue9) to measure circulating BNP peptides. Briefly, after capturing BNP peptides with an antibody raised against the ring region of BNP(1–32) (an antibody routinely used in a commercial BNP assay available from Shionogi) *(26)* bound to magnetic beads, captured BNP peptides were eluted and then detected by MALDI-TOF mass spectrometry (Axima CFR Plus and Axima Confidence, Shimadzu Corp.). Results of coronary angiograms were not made available at time of measurement. The analytical measurement range of the assay was approximately 20–3000 pg/mL. Within-run reproducibility as a measure of analytic precision showed a CV between 7.4% and 8.8% (see online Supplemental Table 1).

STATISTICAL ANALYSIS

We analyzed continuous data, expressed as median with interquartile ranges, by the Wilcoxon rank-sum test to compare medians of values and discrete variables with the Fisher exact test. We used multivariate logistic regression analysis to determine variables associated with restenosis. For multivariable models, a

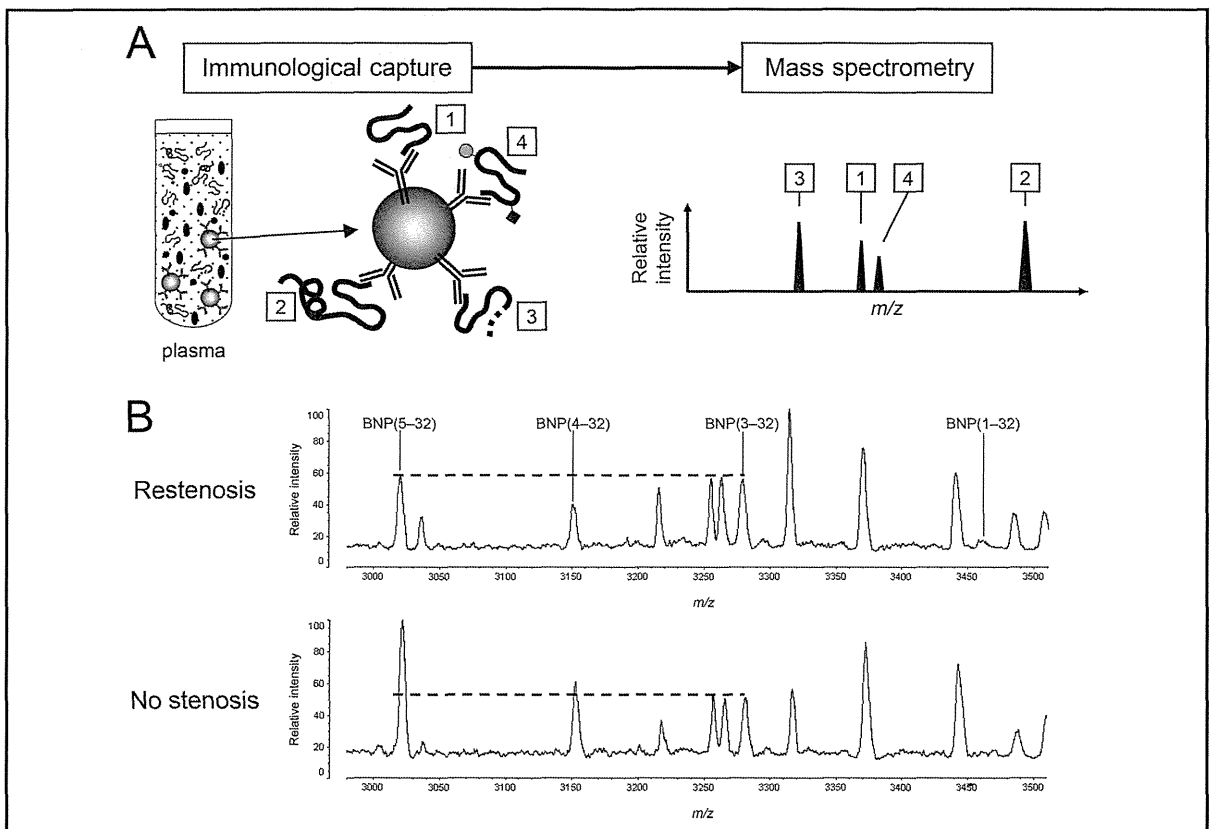

Fig. 1. Mass spectrometry–based immunoassay.

(A), Principle of MS-IA. BNP monoclonal antibodies bound to magnetic beads capture molecular heterogeneity of BNP (1, mature form; 2, premature form; 3, processed form; 4, posttranslationally modified form) in plasma. Mass spectrometric analysis distinguishes the captured molecules in terms of their molecular weight (*m/z*). (B), Mass spectra from 2 representative patients with (upper) and without (lower) restenosis.

stepwise variable selection was performed starting with all of the variables from the univariate model that had a *P* value of <0.2. The final model was generated with backward stepwise logistic regression (*P* to leave: 0.05) (note that a forward stepwise model gave the same results). The final model included only variables that had a *P* value of <0.05. We determined ROC curves, standard diagnostic sensitivity and specificity, likelihood ratios, and predictive value to evaluate diagnostic performance. All statistical analyses were performed with JMP version 8.0.2 (SAS Institute) and MedCalc version 12.3 (MedCalc Software). A 2-tailed $P < 0.05$ was considered statistically significant.

Results

MASS SPECTROMETRY IMMUNOASSAY FOR DETECTION OF CIRCULATING PROCESSED FORMS OF BNP

Because currently available conventional immunoassays cannot discriminate individual processed BNP peptides, we developed a mass spectrometry–based detection method combined with immunocapture by commercial anti-BNP antibodies to detect processed forms of BNP in the circulation, as shown in Fig. 1A. The assay consisted of 2 steps: the first involved immunocapture in which all forms of circulating BNP were captured by anti-BNP monoclonal antibody bound to magnetic beads; the second step involved analysis by mass spectrometry in which captured BNP was eluted from the magnetic beads and analyzed with MALDI-TOF mass spectrometry (further details on the methodology can be found in online Supplemental Text 1).

By use of this method, we detected 3 major forms of BNP: BNP(3–32), BNP(4–32), and BNP(5–32), numbered as amino acids from the amino-terminal end of the 32-amino acid BNP (Fig. 1B). Of the 3 forms, BNP(5–32) was pursued further, as initial measurements showed reduced concentrations of this peptide in patients with restenosis (Fig. 1B). An index peptide

Table 1. Patient characteristics and demographics.[a]

	Factors associated with restenosis (cross-sectional study)			
	Total	No-stenosis	Restenosis	P[b]
n	105	83	22	
Age, years	70 (63–76)	71 (63–77)	69 (66–72)	0.41
Male sex	66 (63)	55 (66)	11 (50)	0.21
Coexisting conditions				
Hypertension	90 (86)	73 (88)	17 (77)	0.30
Diabetes mellitus	65 (62)	52 (63)	13 (59)	0.81
Smoking	71 (68)	56 (67)	15 (68)	1.00
Laboratory values				
Total BNP, pg/mL	51.9 (37.5–83.7)	54.0 (37.5–90.8)	48.1 (31.1–71.3)	0.29
Creatinine, mg/dL	0.83 (0.70–0.94)	0.84 (0.71–0.96)	0.78 (0.65–0.89)	0.15
CRP, mg/L	0.5 (0.3–1.2)	0.5 (0.3–1.2)	0.6 (0.3–1.2)	0.50
Ratio of total cholesterol to HDL cholesterol	3.1 (2.6–3.9)	3.1 (2.5–3.9)	3.1 (2.8–3.8)	0.34
Total cholesterol, mg/dL	170.5 (152.5–190.5)	169.0 (153.9–189.3)	176.0 (149.0–197.0)	0.82
HDL cholesterol, mg/dL	53.2 (44.5–68.6)	53.3 (44.5–68.9)	50.5 (41.5–65.0)	0.63
Triglycerides, mg/dL	134.0 (85.5–184.8)	135.0 (89.0–189.0)	117.0 (77.0–178.5)	0.39
LDL cholesterol, mg/dL	88.5 (78.3–103.5)	87.0 (76.0–102.0)	96.0 (84.0–107.5)	0.09
Systolic blood pressure, mmHg	128.0 (115.0–140.0)	128.0 (112.8–140.0)	128.0 (116.0–142.0)	0.90
Diastolic blood pressure, mmHg	68.0 (60.0–78.5)	68.0 (60.0–78.0)	66.0 (58.0–80.0)	0.58
BNP(5–32)/BNP(3–32)	1.35 (1.19–1.55)	1.43 (1.22–1.61)	1.19 (1.11–1.34)	<0.001
%DS by QCA (%)[c]	14.43 (10.26–25.54)	12.68 (9.18–17.14)	65.52 (59.22–70.54)	<0.001
Lesion length, mm	17.20 (12.58–22.45)	17.47 (13.42–21.89)	14.02 (11.12–24.83)	0.28
Lipid-lowering agents	84 (77)	65 (78)	19 (86)	0.55
Antihypertensive treatment	93 (90)	72 (88)	21 (100)	0.21
Drug-eluting stent	79 (75)	69 (83)	10 (45)	<0.001

[a] Data are median (IQR) or n (%).
[b] P values were determined by the Fisher exact test for discrete variables and the Wilcoxon rank-sum test for continuous variables.
[c] %DS, percent diameter stenosis; QCA, quantitative coronary angiography.

to serve as an internal control to quantify concentrations of BNP(5–32) was needed, but because the full-length peptide, BNP(1–32), was detected in only minute amounts in contrast to BNP(3–32), which was present at higher stable concentrations, an arbitrary index of the ratio of BNP(5–32) to BNP(3–32) was used for further analytical purposes.

DIAGNOSTIC IMPLICATIONS OF PROCESSED FORMS OF BNP

Of the 105 patients enrolled (Table 1 and online Supplemental Table 2), 63% were male (n = 66) and the median age was 70 years [interquartile range (IQR) 63–76]. Comorbid coronary risk factors included hypertension in 90 cases (86%), diabetes mellitus in 65 cases (62%), and smoking in 71 cases (68%). Serum creatinine was 0.83 mg/dL (IQR 0.70–0.94) [73.4 μmol/L (IQR 61.4–83.1)]; C-reactive protein (CRP) was 0.5 mg/L (IQR 0.3–1.2); HDL cholesterol was 53.2 mg/dL (IQR 44.5–68.6) [1.4 mmol/L (IQR 1.2–1.8)]; LDL cholesterol was 88.5 mg/dL (IQR 78.3–103.5) [2.3 mmol/L (IQR 2.0–2.7)]; and BNP was 51.9 pg/mL (IQR 37.5–83.7). 75% of patients (79 cases) were treated with drug-eluting stents, and angiographic outcome at follow-up CAG showed 22 cases of defined restenosis (21% overall, 13% for drug-eluting stents).

The BNP(5–32)/BNP(3–32) ratio was significantly lower in patients with restenosis at time of follow-up CAG (restenosis 1.19, IQR 1.11–1.34, n = 22, vs without restenosis 1.43, IQR 1.22–1.61, n = 83; $P < 0.001$) (Table 1 and Fig. 2A). Notably, total BNP concentrations as measured with a standard commercial immunoassay did not show association with restenosis (Table 1 and Fig. 2B). Reference median concen-

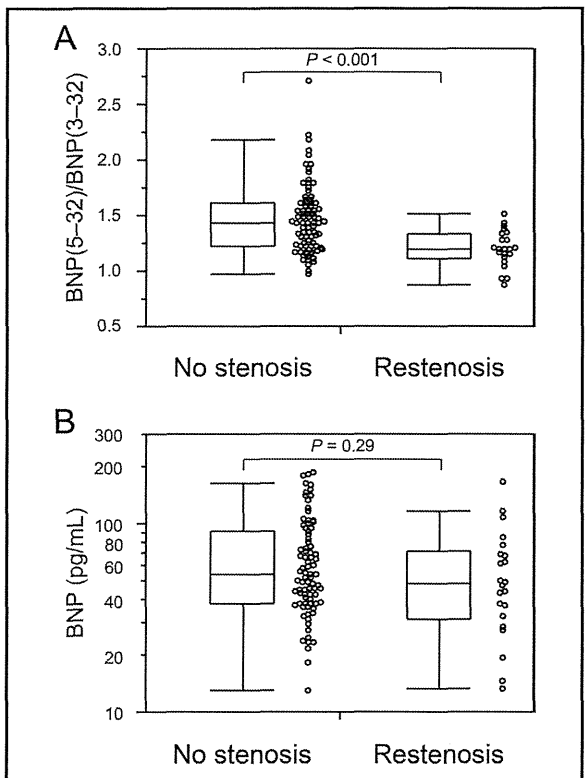

Fig. 2. BNP(5–32)/BNP(3–32) ratio and total concentrations of BNP in restenosis.

Association between restenosis diagnosed by CAG and BNP(5–32)/BNP(3–32) ratio (A) and total concentrations of BNP in representative patients with and without restenosis (B) in the cross-sectional study. No stenosis, n = 83; restenosis, n = 22. Boxes represent IQR, and the horizontal line in each box represents the median.

trations of BNP and BNP(5–32)/BNP(3–32) ratio in the present study were 57.5 pg/mL (IQR 39.5–94.2, n = 66) and 1.43 (IQR 1.28–1.72, n = 66), respectively.

ROC analysis of the diagnostic accuracy of the BNP(5–32)/BNP(3–32) ratio for those with presence of restenosis showed an area under the curve of 0.775 (95% CI 0.683–0.851), and the optimal cutoff value for discrimination of stenosis was 1.41 (sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio were 91%, 54%, 1.99, and 0.17, respectively) (see online Supplemental Table 3 and Supplemental Fig. 1). Sensitivity and specificity as well as negative and positive likelihood ratios in addition to positive and negative predictive values are shown in online Supplemental Table S3. Of interest, a negative likelihood ratio of <0.1 allowing for reliable rule-out *(27)* was attained at a ratio of 1.52, with both sensitivity and negative predictive value of 100%. Thus, measuring BNP processed forms as the BNP(5–32)/BNP(3–32) ratio had diagnostic value for ruling out restenosis.

We used univariate and multivariate analyses to examine the association of the BNP(5–32)/BNP(3–32) ratio with restenosis, taking into account the measured concentrations of other laboratory blood tests (total BNP, serum creatinine, CRP, ratio of total cholesterol to HDL cholesterol, total cholesterol, HDL cholesterol, triglycerides, and LDL cholesterol), risk factors (age, sex, hypertension, diabetes mellitus, smoking, use of lipid-lowering agents, and antihypertensive treatment), systolic and diastolic blood pressure, lesion length, and drug-eluting stent use for PCI. The BNP(5–32)/BNP(3–32) ratio [odds ratio (OR) 0.63; 95% CI 0.45–0.83; $P < 0.001$] and failure to use a drug-eluting stent (OR 4.20; 95% CI 1.40–12.99; $P = 0.011$) were significantly and independently associated with restenosis (Table 2). OR analysis showed that there was a 1.59-fold reduction in likelihood for restenosis with each 0.1 U increase in the BNP(5–32)/BNP(3–32) ratio.

Discussion

Peptide processing has become increasingly recognized as important not only in metabolism of peptides but also in regulation of various pathologies, particularly since peptide processing has become the target of therapeutic intervention with pharmaceutical development of protease inhibitors in treatment of disease [e.g., DPP-IV inhibitors *(22, 23)*]. Recent studies have also focused on the possible exploitation of peptide processing in diagnosis of Alzheimer disease *(20)* and a potential role in ischemic heart disease *(17, 18)*. In the present study, we focused on the bioactive cardiac hormone BNP, whose circulating concentrations are reflective of pathogenic activity and have been clinically used for diagnostic purposes, and showed that its processed forms are strongly associated with the condition of restenosis in ischemic heart disease. Methods to measure these peptide forms were developed using mass spectrometry–based detection combined with immunocapture, because conventional immunoassay methods are not able to discriminate the different forms. Our initial experience shows that measurement of BNP processing with this method is of potential use to diagnose restenosis.

We found that 3 major processed forms of circulating BNP—BNP(3–32), BNP(4–32), and BNP(5–32)—in addition to minute amounts of full-length BNP(1–32), were those primarily detected in the circulation in ischemic heart disease. Markedly lower concentrations of BNP(5–32) were seen in patients with restenosis at time of follow-up CAG. OR analysis showed that there was a 1.59-fold reduction in likeli-

Table 2. Univariate and multivariate analysis of factors associated with restenosis.

	Univariate analysis		Multivariate analysis[a]	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	0.99 (0.94–1.05)	0.74		
Male sex	0.51 (0.19–1.33)	0.17		
Hypertension	0.47 (0.14–1.65)	0.22		
Diabetes mellitus	0.86 (0.33–2.31)	0.76		
Smoking	1.03 (0.39–2.98)	0.95		
BNP	0.99 (0.98–1.00)	0.23		
Creatinine	0.09 (0.004–1.28)	0.08		
CRP	0.99 (0.86–1.08)	0.91		
Ratio of total cholesterol to HDL cholesterol	1.17 (0.69–1.93)	0.55		
Total cholesterol	1.00 (0.98–1.01)	0.53		
HDL cholesterol	0.99 (0.96–1.02)	0.70		
Triglycerides	1.00 (0.99–1.00)	0.26		
LDL cholesterol	1.01 (0.99–1.03)	0.44		
Systolic blood pressure	1.00 (0.97–1.03)	0.90		
Diastolic blood pressure	0.99 (0.95–1.03)	0.65		
BNP(5–32)/BNP(3–32)	0.60 (0.43–0.78)	<0.001	0.63 (0.45–0.83)	<0.001
Lesion length	0.97 (0.91–1.03)	0.39		
Lipid-lowering agents	1.75 (0.52–8.05)	0.38		
Antihypertensive treatment	3.21 (0.57–60.32)	0.21		
Drug-eluting stent not used	5.91 (2.16–16.80)	<0.001	4.20 (1.40–12.99)	0.011

[a] Only variables from the univariate analysis that had a *P* value of <0.2 were retained in the multivariate model. The final model included only variables that had a *P* value of <0.05.

hood for presence of restenosis with each 0.1 U increase in the BNP(5–32)/BNP(3–32) ratio. Importantly, this ratio of the concentrations of processed forms of BNP was to be found useful as a new biomarker to rule out the presence of restenosis at cutoff concentrations of 1.52.

Our results suggest that processed forms of BNP, especially BNP(5–32), may reflect the pathophysiological process involved in restenosis. BNP is synthesized as preproBNP(1–134), which results in proBNP(1–108) after the removal of a 26-aa signal peptide. ProBNP(1–108) is cleaved to a biologically inactive amino-terminal NT-proBNP(1–76) and active BNP(1–32) *(13)*. A cardiac transmembrane serine protease, corin, and a ubiquitous serine protease, furin, are currently proposed as possible convertases *(16, 28, 29)*. Recently, other processed forms of BNP, including BNP(3–32), BNP(4–32), and BNP(5–32), have been detected in plasma from heart failure patients in the presence of protease inhibitors benzamidine (as a trypsin, plasmin, thrombin inhibitor) and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (as an inhibitor for serine protease such as DPP-IV) to minimize the effect of protease degradation *(15)*. Of the 3 processed forms of BNP, BNP(3–32) has been reported to be processed from BNP(1–32) by DPP-IV *(14)*. BNP(4–32) has been reported to be processed by corin from proBNP, not from BNP(1–32) *(16)*. Additionally, BNP(5–32) has been reported to be processed possibly from BNP(1–32) by neutral endopeptidase *(30)*, but another study has reported that BNP(1–32) is resistant to neutral endopeptidase–mediated cleavage *(14)*. Further, a recent study has reported that human proBNP injected into rats is processed into BNP(5–32) *(31)*, thus indicating that BNP(5–32) may be processed by an unknown protease in rats. Thus, the underlying pathologic mechanisms of BNP processing are thought to involve the combined actions of membrane-bound-type protease(s) such as neutral endopeptidases and dipeptidyl peptidases, but the precise underlying mechanisms of action are not understood. Pathogenic regulation of peptidase activity in disease states likely defines the proportion of BNP forms present in the circulation, and will be a topic of further investigation in the future.

Other attempts including some by our group to develop biomarkers of restenosis by use of interleukin-6 *(32)*, oxidized LDL cholesterol markers *(33)*, LDL cholesterol *(34)*, HDL cholesterol *(35)*, CRP *(36–38)*, adiponectin *(39)*, and their combinations have not proven clinically useful. Clinical algorithms also are not reliable *(4, 5)*. Reduced relative concentrations of BNP(5–32), as measured with an analytical ratio of BNP(5–32)/BNP(3–32), were found to be strongly associated with presence of restenosis in our cross-sectional study. To our knowledge, diagnostic performance of the magnitude described in the present study has not been achieved by any other biomarker to date. Importantly, a rule-out biomarker has not been available for this condition to assist in risk stratification of patients.

The described biomarker might aid in identifying patients with less risk of restenosis after a PCI procedure. A tool for noninvasive identification of patients without restenosis after a PCI procedure would be helpful to reduce the burden of performing routine follow-up CAG. It would also be of merit in those settings in which follow-up CAG is not routinely done but is reserved as a tool to assist in ruling out the presence of restenosis when assessing patients with ambiguous chest pain after PCI. It is important to note that restenosis had been generally thought to be associated with relatively benign outcome, but recent evidence suggests that it is associated with myocardial damage and adverse clinical outcome (30% to 60% present with acute coronary syndrome, 5% present with ST-elevation myocardial infarction) [see *(40)* for review]. Therefore, given this need to identify patients at risk for restenosis, a noninvasive biomarker would be a welcome tool in management of the condition.

Longitudinal studies to determine the prognostic value of processed forms of BNP and clinical studies to address the association of these novel biomarkers with coronary events will be of further interest and are presently ongoing. The limitations of the current study include the need for further large-scale studies at multiple centers to validate the present findings. Additionally, there is need for studies that explore combined use of clinical algorithms with this and possibly other biomarkers to more accurately assess risk of restenosis. Further modification of this technology will be necessary to make this method or its derivatives more widely available for patient care.

In summary, we provide our initial experience with a newly developed method to measure processed forms of BNP as a biomarker for risk assessment in patients undergoing PCI for ruling out restenosis.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: H. Fujimoto, Shimadzu Corporation.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: T. Suzuki, research grants from the Ministry of Health, Labour and Welfare of Japan for Research on Medical Device Development and for Research on Biological Markers for New Drug Development; Grants-in-Aid for Scientific Research in Priority Areas (B)(23390204) and for Translational Systems Biology and Medicine Initiative (TSBMI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the Japan Society for the Promotion of Science through its Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program); R. Nagai, Japan Society for the Promotion of Science through its Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program).
Expert Testimony: None declared.
Patents: H. Fujimoto, WO2010/023749; T. Suzuki, WO2010/023749.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: The authors thank Shionogi & Co. (Osaka, Japan) for kindly providing monoclonal BNP antibody (KY-hBNP II).

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Supplemental Data:

Text S1. Detailed description of the mass spectrometry-based immunoassay (MS-IA) procedure.

Mass Spectrometry-based Immunoassay

A mass spectrometry-based immunoassay (MS-IA) procedure was developed to measure circulating BNP processed forms. The assay consisted of two steps; the first step involved an immuno-capture step in which all forms of circulating BNP were captured by anti-BNP monoclonal antibody-bound magnetic beads, and the second step involved analysis by mass spectrometry in which captured BNP was eluted from magnetic beads and analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

Magnetic beads (5 μL suspension per 330 μL sample) with covalently bound sheep anti-mouse IgG (Dynabeads M-280 Sheep anti-mouse IgG, Invitrogen Dynal AS, Oslo, Norway) were pre-washed three times with 1 mL of phosphate-buffered saline (PBS, pH 7.4) then pelleted for 1 min using a magnetic particle concentrator (Dynal MPC-S, Invitrogen Dynal AS, Oslo, Norway). After removal of PBS, the beads were incubated with 7 μg of the monoclonal antibody, KY-hBNP II (kindly provided by Shionogi & Co., Ltd., Osaka, Japan) (1), in 1 mL of PBS for 1 hour at room temperature on a rotary shaker. The remaining unbound antibody was removed by washing five times with 1 mL of PBS. For control experiments, beads were incubated without antibody. 330 μL of plasma, thawed at 37°C in a water bath, was added to the antibody-coated beads in PBS containing 0.1% zwittergent 3–16 detergent (Calbiochem, Darmstadt, Germany) in duplicate (final volume of 1 mL), and then incubated for an additional 1 hour at room temperature. For control experiments, plasma was incubated with beads not coated

with antibody. Beads were pelleted for 1 min using a magnetic particle concentrator and then further washed four times with PBS and two times with 20 mM ammonium bicarbonate. Beads were then washed with 5 μL of 0.01% trifluoroacetic acid (TFA) in water. Extracted BNP was eluted by adding 2.5 μL of 0.02% TFA in water. After vortexing for 10 seconds, the beads were pelleted by a magnetic particle concentrator. Eluents were spotted onto a MALDI target plate.

One microliter of matrix solution (10 mg/mL 2,5 dihydroxybenzoic acid in 0.05% TFA and 50% acetonitrile : 5 mg/mL α-cyano-4-hydroxycinnamic acid in 0.05% TFA and 50% acetonitrile = 1 : 1) was added to the MALDI plate and the sample was then left to completely dry in air on a 37°C heat block. MALDI-TOF MS measurements were performed using an AXIMA-CFR plus and AXIMA confidence (Shimadzu Corporation, Kyoto, Japan) operating in linear mode. The spectra represent an average of more than 400 profiles (5 shots/profile) recorded up to 20,000 Da and calibrated using an external calibration standard (adrenocorticotropic hormone 18–39 fragment, m/z 2466.72 [average]; and oxidized insulin B chain, m/z 3496.96 [average]). Two spectra were obtained from each sample and analyzed in duplicate. All mass spectra were analyzed by Launchpad ver. 2.4.0 software for AXIMA-CFR plus and ver. 2.8 for AXIMA confidence (Shimadzu Corporation, Kyoto, Japan) using baseline subtraction and then smoothed with a 20-width average method. All reported m/z are the average peaks. Results of coronary angiograms were not made available at time of measurement.

Based on preliminary analysis of patients showing three predominant alternate forms of BNP – BNP(3–32), BNP(4–32) and BNP(5–32) – in the circulation, synthesized peptides of these forms and commercially available BNP(1–32) (all peptides from Peptide Institute Inc., Osaka, Japan) were added at equimolar concentrations to normal plasma from healthy volunteers to evaluate antibody specificity as well as sensitivity and reproducibility of measurements. The

antibody was able to capture these three different forms of BNPs in a specific manner (Figure S2). The measuring range of the assay was around 20 to 3,000 pg/mL. Assay sensitivity of 2.5 fmol per sample for each peptide which corresponds to approximately 8.8 pg of BNP(1–32), 8.3 pg of BNP(3–32), 7.9 pg of BNP (4–32), and 7.6 pg of BNP (5–32) was achieved.

To evaluate intra- and inter-assay precision, we prepared normal plasma from healthy volunteers spiked with known amounts of BNPs and then made measurements over a period of three consecutive days. Intra-assay precision (coefficient of variation) was 7.4% and 8.8% at 2.5 fmol and 10 fmol, respectively, and inter-assay precision (coefficient of variation) for triplicate analyses over three days was 9.8% and 11.8% at 2.5 fmol and 10 fmol, respectively (Table S1).

BNP is known to be degraded by proteases in the circulation and plasma collection conditions are crucial to preserve the molecular heterogeneity of BNP (2, 3). The degradation pattern of commercially available BNP(1–32) spiked at 200 fmol/mL (approximately 700 pg/mL) into whole blood collected in serum or plasma (ethylenediaminetetraacetate-2Na and aprotinin) from a healthy volunteer and patient was tested (Figure S3A–D). Rapid degradation of intact BNP(1–32) was observed both in serum and plasma samples. After 15 minutes from blood sampling, new peaks that could be assigned to BNP(3–32) appeared. For serum samples, BNP(3–32) continued to be degraded to other BNP forms such as BNP(4–32), BNP(1–29), BNP(3–30), BNP(3–29), BNP(4–29), BNP(5–29) and BNP(6–29) (Figure S3A, C). In contrast, for plasma samples, BNP(3–32) did not continue to be degraded up to 6 hours at 4°C except that BNP(4–32) could be faintly detected (Figure S3B, D). Further, endogenous BNP in patient samples showed degradation of endogenous BNP in serum samples with appearance of signals for BNP(3–29), BNP(5–29) and BNP(6–29) which did not occur in plasma (Figure S3F). Processed endogenous BNP was not detectable in plasma from a healthy volunteer (Figure S3E).

Detected signals are summarized in Figure S3G. Note that BNP(5–32) was only detected in endogenous BNP in patient samples, and to be stable in plasma without further degradation. It is, therefore, unlikely that BNP(5–32) is an artifact of ex vivo degradation of BNP(3–32).

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Table S1. Intra- and Inter-assay Precision

	BNP(5–32)/BNP(3–32)	
	Intra-assay	**Inter-assay**
2.5 fmol		
Replicates	3	9
Mean	1.08	1.02
Standard deviation	0.08	0.10
Coefficient of variation, %	7.4	9.8
10 fmol		
Replicates	3	9
Mean	1.02	0.93
Standard deviation	0.09	0.11
Coefficient of variation, %	8.8	11.8

Table S2. Diagnostic Performance of the BNP(5–32)/BNP(3–32) Ratio for Presence of Restenosis

Cut-off value	Sensitivity, %	95% CI	Specificity, %	95% CI	PLR	NLR	PPV, %[a]	NPV, %[a]
0.87	0.0	0.0–15.4	100.0	95.7–100.0	-	1.00	-	79.0
0.93	13.6	2.9–34.9	100.0	95.7–100.0	-	0.86	100.0	81.4
1.00	13.6	2.9–34.9	97.6	91.6–99.7	5.66	0.88	60.0	81.0
1.04	18.2	5.2–40.3	97.6	91.6–99.7	7.55	0.84	66.7	81.8
1.05	18.2	5.2–40.3	96.4	89.8–99.2	5.03	0.85	57.1	81.6
1.09	22.7	7.8–45.4	95.2	88.1–98.7	4.72	0.81	55.6	82.3
1.11	22.7	7.8–45.4	91.6	83.4–96.5	2.69	0.84	41.7	81.7
1.12	27.3	10.7–50.2	90.4	81.9–95.7	2.83	0.80	42.9	82.4
1.14	27.3	10.7–50.2	89.2	80.4–94.9	2.52	0.82	40.0	82.2
1.15	36.4	17.2–59.3	86.8	77.5–93.2	2.74	0.73	42.1	83.7
1.16	40.9	20.7–63.6	81.9	72.0–89.5	2.26	0.72	37.5	84.0
1.18	40.9	20.7–63.6	80.7	70.6–88.6	2.12	0.73	36.0	83.7
1.19	54.6	32.2–75.6	79.5	69.2–87.6	2.66	0.57	41.4	86.8
1.20	63.6	40.7–82.8	78.3	67.9–86.6	2.93	0.46	43.7	89.0
1.25	63.6	40.7–82.8	69.9	58.8–79.5	2.11	0.52	35.9	87.9
1.28	72.7	49.8–89.3	68.7	57.6–78.4	2.32	0.40	38.1	90.5
1.32	72.7	49.8–89.3	63.9	52.6–74.1	2.01	0.43	34.8	89.8
1.33	77.3	54.6–92.2	61.4	50.1–71.9	2.00	0.37	34.7	91.1
1.35	81.8	59.7–94.8	57.8	46.5–68.6	1.94	0.31	34.0	92.3
1.37	86.4	65.1–97.1	57.8	46.5–68.6	2.05	0.24	35.2	94.1
1.39	86.4	65.1–97.1	55.4	44.1–66.3	1.94	0.25	33.9	93.9
1.41	90.9	70.8–98.9	54.2	42.9–65.2	1.99	0.17	34.5	95.7
1.43	95.5	77.2–99.9	48.2	37.1–59.4	1.84	0.09	32.8	97.6
1.49	95.5	77.2–99.9	39.8	29.2–51.1	1.58	0.11	29.6	97.1
1.52	100.0	84.6–100.0	36.1	25.9–47.4	1.57	0.00	29.3	100.0
2.70	100.0	84.6–100.0	0.0	0.0–4.3	1.00	-	21.0	-

[a] Prevalence rate of 10% for restenosis (drug-eluting stents) was used for predictive value calculations (ref. 4).

CI, confidence interval; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

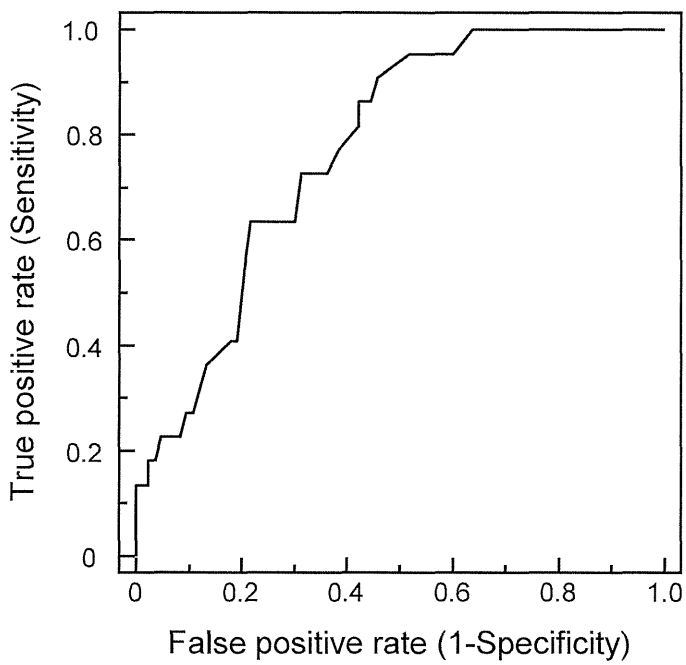


Figure S1. Diagnostic performance of the developed mass spectrometry-based immunoassay procedure for diagnosis for presence of restenosis as shown by ROC curve analysis. Area under the curve, 0.775 (95% CI, 0.683 to 0.851).

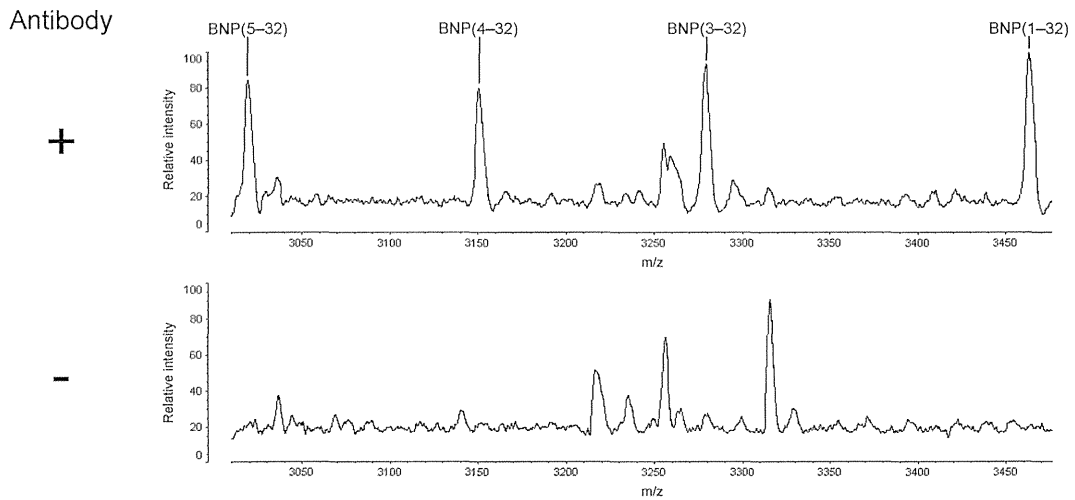
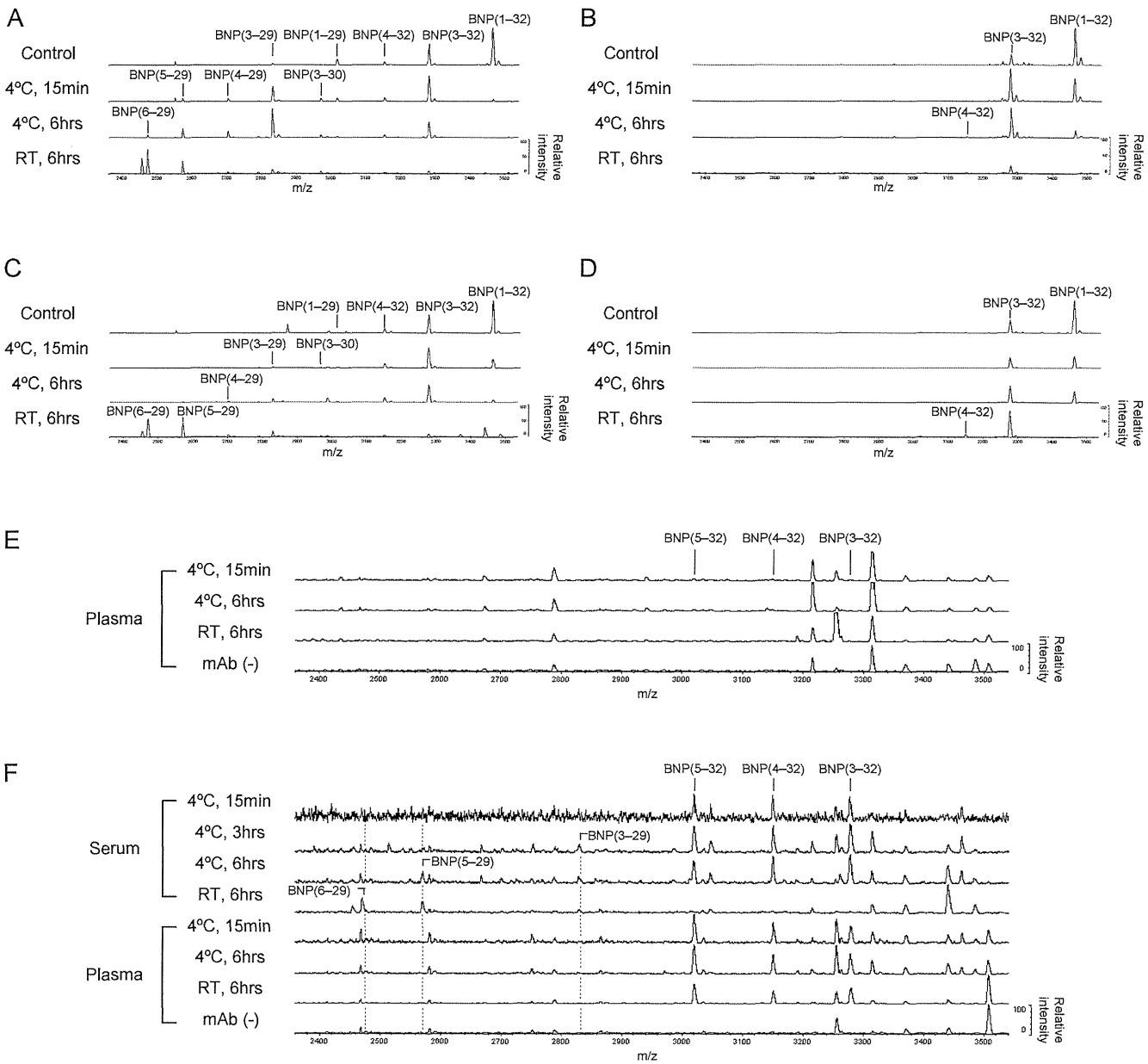


Figure S2. Specificity of the BNP monoclonal antibody (KY-hBNP II). KY-hBNP II recognized various types of BNP molecules including BNP(1–32), BNP(3–32), BNP(4–32) and BNP(5–32).



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Detected BNP signals at 4°C, 6hrs			1–32	3–32	4–32	5–32	1–29	3–30	3–29	4–29	5–29	6–29
Spiked BNP(1–32)	Healthy volunteer	Serum (A)	-	+	+	-	+	+	+	+	+	+
		Plasma (B)	+	+	+	-	-	-	-	-	-	-
	Patient	Serum (C)	+	+	+	-	+	+	+	+	+	-
		Plasma (D)	+	+	-	-	-	-	-	-	-	-
Endogenous BNP	Healthy volunteer	Plasma (E)	-	-	-	-	-	-	-	-	-	-
	Patient	Serum (F)	-	+	+	+	-	-	+	-	+	+
		Plasma (F)	-	+	+	+	-	-	-	-	-	-

Figure S3. Processing of BNP. Time course for BNP(1–32) proteolysis in serum (**A, C**) and plasma (**B, D**). Plasma sampling tube contains ethylenediaminetetraacetate-2Na and aprotinin. Commercially available BNP(1–32) was added at 200 fmol/mL immediately after drawing blood from a healthy volunteer (**A, B**) and from a patient (**C, D**). (**E**) Processed endogenous BNP was not detectable in plasma from a healthy volunteer. (**F**) The degradation patterns of endogenous BNP in serum (upper 4 panels) and plasma (lower 4 panels) from a patient. Detected signals are summarized in (**G**). + indicates signals which were detected, and – indicates signals which were not detected.

新しい動脈硬化のバイオマーカー開発——新しいプロテオーム技術を用いて

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はじめに

われわれは動脈硬化疾患のバイオマーカー探索を主な研究テーマとしている．急性疾患として，平滑筋ミオシン，クレアチンキナーゼBBアイソザイム，最近ではカルポニン（平滑筋トロポニン様蛋白）およびD-ダイマーが急性大動脈解離のバイオマーカーであることを相次いで明らかにしてきた．慢性疾患として，酸化LDLの測定系を開発した．これらの酵素免疫法を用いたアッセイの一部はすでに一般臨床の現場で用いられているが，最近，この十年間においてはプロテオミクス（質量分析）等の新しい技術が注目されている．

われわれはこの分野に最新の技術（免疫質量分析法，プロテインチップ法）を導入し，その臨床応用を行い貢献してきた．一例として，冠動脈再狭窄の除外診断に役立つ有望なバイオマーカーを開発した．このアッセイは心臓特異的蛋白がプロセスされたペプチド産物を質量分析計で測定するものだが，冠動脈再狭窄，すなわち強い冠動脈疾患患者におけるペプチダーゼ反応を検出するものである．予備試験では感度100％であり，再狭窄の除外診断として信頼できる結果を得た．本バイオマーカーを用いた再狭窄の予知が可能か検討したところ，同様の結果を得た．予備試験結果ではあるが，本結果は期待できるものであり，はじめてのプロテオームを用いたバイオマーカーとして臨床応用可能なバイオマーカーとなる見込みである．

本稿の前半では，酸化LDLを中心とした動脈硬化におけるメカニズムおよびバイオマーカーについて，後半では，最近のプロテオミクス解析技術を用いたエピジェネティクス研究の成果と，同技術を心血管病態の解析（バイオマーカー等）を中心とした医療に応用する試みを中心に概説する．

動脈硬化形成に重要な蛋白の変性

動脈硬化は多因子が関与する複雑な病態である．脂質異常のみならず，血管内皮細胞機能障害，血管平滑筋細胞障害，脂肪細胞の機能不全，炎症，石灰化などさまざまな要素が関係する．中でも，最近，社会的にも注目を浴びるメタボリックシンドロームを背景として発症する心血管疾患等の生活習慣病は，その発症前に非常に長い潜伏期間を有するが，この間に経年的な蛋白質の変性病態が進行する．このように，長い潜伏期間において蛋白質は，発現調節，プロセシング，化学修飾，相互作用等の多段階にわたる制御を受けるが，病態・老化変性等の場合，さらに経時的変化による制御が加わる（図1）．よって，蛋白質の

[Key words]　バイオマーカー，急性大動脈解離，酸化LDL，冠動脈再狭窄

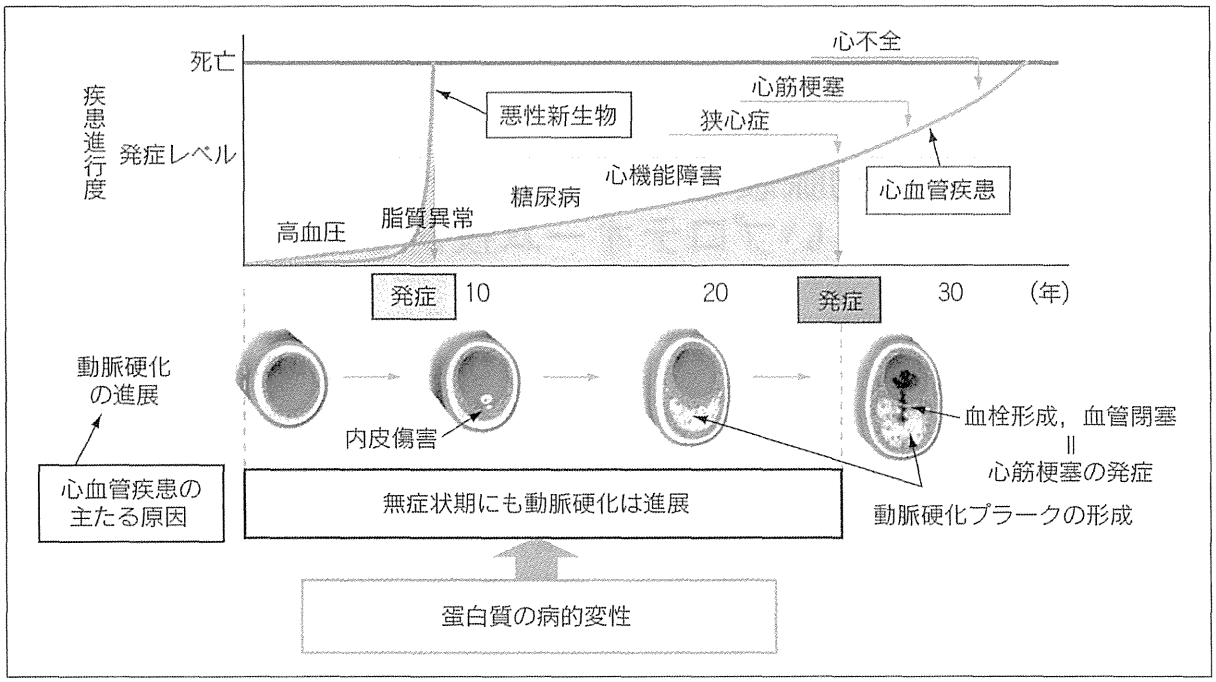

図1　心血管病の形成過程と蛋白質の変性
心血管疾患等の生活習慣病は，発症前に非常に長い潜伏期間を有するが，この間に経年的な蛋白質の変性が進行する．

質的・量的変化の時空間的な制御の理解は，ヒトの多様性や疾患発症の個人差をはじめテーラーメード（個別化）医療の解明の鍵となると考えられる．とくに成人後に罹る疾病（心血管疾患，生活習慣病）については，発症時期の情報として蛋白質の動態ならびにその変化に関する情報は重要である．

多様なLDLの変性と酸化LDLの測定

LDLは540 kDの巨大分子であるアポ蛋白B（アポB）とコレステロール，リン脂質，中性脂肪などの脂質から構成され，活性酸素により不飽和脂質の過酸化が生じる．この過程で，脂肪酸の分解産物として各種のアルデヒドやケトンが生じるが，これらの反応性分子がアポBを修飾し，LDLを変性させた結果，酸化LDLが生じる．すなわち，酸化LDLはLDLが多様な酸化修飾を受けているため，不均一な成分構成からなる（図2）．

酸化LDLの臨床的意義を明らかにするうえで，末梢血中の酸化LDLの濃度を測定することは心血管疾患のリスク管理のうえで有用性が高い．最近，LDLにおけるホスファチジルコリンの酸化を抗酸化ホスファチジルコリン抗体と抗アポB抗体を用いたサンドイッチELISA法で測定する系が開発された[1~4]．この測定法によると，血中の酸化LDL値は冠動脈疾患患者で有意に高値であり，総コレステロール（TC），中性脂肪（TG），LDLコレステロールなどの従来の脂質マーカーに比較しても感度・特異度ともっとも高い相関を示した[3]．とくに，若年男性において顕著な相関があり，若年男性の心血管イベント抑制を目標とした早期介入に役立つものと思われる．その他，いくつかの横断的な研究により，臨床的な冠動脈疾患とLDLの酸化修飾の関与が示された．酸化LDL値は狭心症（不安定・安定いずれも）患者群と比較し，心筋梗塞患者群で高値を示した[1,4]．

脂質酸化物のうちもっとも代表的なものがLDLであり，量も豊富である．これがアポBのリジン残基に結合したものが，マロンジアルデヒド化