せん

この試験で得られた成績は、医学雑誌などに公表されることがありますが、あなたの名前などの個人的情報は一切わからないようにしますので、プライバシーは守られます。

また、この試験で得られたデータが、本試験の目的以外に学会や論文発表等にも使用されることもあります。その場合にもプライバシーは守られます。

１４．　この試験への参加に同意された場合は、次の点を守って下さい

本試験への参加に際しては、通院期日を守ってください。また、所定の検査をお受けください。他科・他院を受診する際は、必ず試験に参加していることを当該医師に告げるとともに、可能な限り事前に試験担当医師に相談してください。また、事後に必ず試験担当医師にお知らせください。

１５．　あなたの費用負担について

試験のために特別に行われる検査は、研究費より支払われ、患者さんの負担が増えることはありません。

１６．　知的財産権と利益相反について

本試験の結果が特許権等の知的財産を生み出す可能性がありますが、その場合の知的財産権は研究者もしくは所属する研究機関に帰属します。

本試験の実施に関して、利益相反（起こりうる利害の衝突）が存在しないことを確認しています。

１７．　この担当医師が、あなたを担当致します

（氏名）	（所属機関）	（診療科）	（職名）	（連絡先）
小室一成	東京大学	循環器内科	教授	03-3815-5411（内線 33100）

○鈴木　亨	東京大学	ﾕﾋﾞｷﾀｽ予防医学講座	特任准教授	03-3815-5411（内線 35636）
相澤健一	東京大学	循環器内科	特任助教	03-3815-5411（内線 33117）
澤城大悟	東京大学	ﾕﾋﾞｷﾀｽ予防医学講座	特任助教	03-3815-5411（内線 35636）
石田純一	東京大学	循環器内科	助教	03-3815-5411（内線 33117）
藤田英雄	東京大学	健康空間情報学講座	特任准教授	03-3815-5411（内線 37164）
安東治郎	東京大学	附属病院ﾏﾈｼﾞﾒﾝﾄ領域	講師	03-3815-5411（内線 37151）

（○：試験責任医師）

＜研究事務局＞

東京大学　　循環器内科

相澤健一（研究事務局代表）、

〒113-8655 東京都文京区本郷 7-3-1、

電話：03-3815-5411（内線 35636）、FAX：03-5800-8824

E-mail：aizawak-tky@umin.ac.jp（相澤健一）

１８．　いつでも相談窓口にご相談下さい

あなたがこの試験について知りたいことや、心配なことがありましたら、遠慮なく担当医師または臨床研究支援センターにご相談下さい。ご希望により本試験計画および試験の方法に関する資料の一部を閲覧することも可能です。

東京大学医学部附属病院（代表電話　０３－３８１５－５４１１）

試験担当医師

鈴木　亨	東京大学	ﾕﾋﾞｷﾀｽ予防医学講座	特任准教授	03-3815-5411（内線 35636）
相澤健一	東京大学	循環器内科	特任助教	03-3815-5411（内線 33117）

臨床研究支援センター：月～金８：３０～１７：００（内線３４２９１）

循環器内科当直医師：上記以外の時間帯　03-3815-5411（内線 30215）

患者さん用

同意文書

自主臨床試験課題名：冠動脈カテーテル治療後再狭窄の新規バイオマーカー（BNP 断片比）の探索的臨床的試験

＜説明事項＞

1. はじめに：自主臨床試験について
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3. この試験の方法
4. この試験の予定参加期間
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15. あなたの費用負担について
16. 知的財産権と利益相反について
17. 担当医師
18. 相談窓口

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同意日：平成　　年　　月　　日　　患者ＩＤ：＿＿＿＿＿＿＿＿＿＿

患者氏名：＿＿＿＿＿＿＿＿＿＿（自署）

【医師の署名欄】
私は、上記患者さんに、この自主臨床試験について十分に説明いたしました。

説明日：平成　　年　　月　　日　　所属：＿＿＿＿＿＿＿＿＿＿

氏名：＿＿＿＿＿＿＿＿＿＿（自署）

臨床研究支援センター用

同意文書

自主臨床試験課題名：冠動脈カテーテル治療後再狭窄の新規バイオマーカー（BNP 断片比）の探索的臨床的試験

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患者氏名：＿＿＿＿＿＿＿＿＿＿（自署）

【医師の署名欄】
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説明日：平成　　年　　月　　日　　所属：＿＿＿＿＿＿＿＿＿＿

氏名：＿＿＿＿＿＿＿＿＿＿（自署）

医師用

同意文書

自主臨床試験課題名：冠動脈カテーテル治療後再狭窄の新規バイオマーカー（BNP 断片比）の探索的臨床的試験

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患者氏名：＿＿＿＿＿＿＿＿＿＿（自署）

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氏名：＿＿＿＿＿＿＿＿＿＿（自署）

自主臨床試験・様式第6号
(委員長から病院長および病院長から試験責任医師)

整理番号	P2013048-11Y

Ver.2.3

2014/01/23

試験審査結果通知書

実施医療機関の長
東京大学医学部附属病院
病院長　門脇 孝 殿

臨床試験審査委員会
名称　東京大学医学部附属病院臨床試験審査委員会
所在地 東京都文京区本郷7丁目3番1号
委員長　南学 正臣

審査依頼のあった件についての審査した結果を下記のとおり通知いたします。

記

試験課題名	冠動脈カテーテル治療後再狭窄の新規バイオマーカー（BNP断片比）の探索的臨床試験
審査事項 (審査資料)	■試験の実施の適否 (自主臨床試験および未承認薬等の臨床使用申請書(2014/01/07　付様式第2号)) □試験の継続の適否 □重篤な有害事象 (重篤な有害事象の報告書(西暦 年 月 日付様式第11号)) □安全性情報等 (新たな安全性情報の報告書(西暦 年 月 日付様式第12)) □試験に関する変更 (一部変更申請書(西暦 年 月 日付様式第10)) □緊急の危険を回避するための試験実施計画書からの逸脱 (緊急の危険を回避するための試験実施計画書からの逸脱に関する報告書 (西暦 年 月 日付様式第13)) □継続審査 (試験実施状況報告書(西暦 年 月 日付様式第9)) □その他( )
審査区分	☒ 委員会審査　(審査日: 2014/01/23) □ 迅速審査　(審査終了日:)
審査結果＊	□ 承認　☒ 修正の上で承認　□ 却下　□ 既承認事項の取り消し　□ 保留
指示事項および理由・条件等＊	試験実施計画書および説明文書について、当日提出された修正案のとおり修正すること。 回答書の提出の要否 [☒ 要 □ 否]
備考	

＊未承認薬等の臨床使用の場合は、「(臨床)試験」とあるのを「(臨床)使用」と適宜読み替えるものとする。
＊「修正の上で承認」とは正式な承認ではない。回答書の提出「要」となった場合は、試験開始前に必ず対応すること。
修正が済み文書が必要な場合は、回答書とともに提出すること。

2014/01/23

治験責任医師 ユビキタス予防医学講座・鈴木亨 殿

依頼のあった試験に関する審査事項について上記のとおり決定しましたので通知いたします。

実施医療機関の長
東京大学医学部附属病院
病院長　門脇 孝

自主臨床試験・様式第７号
（試験責任医師から臨床試験審査委員長）（臨床試験審査委員長から病院長）（病院長から試験責任医師）Ver.1.3

西暦 2104 年 1 月 30 日

臨床試験審査委員会の指示事項への回答書

東京大学医学部附属病院臨床試験審査委員会委員長 殿

ユビキタス予防医学講座
試験責任医師
職名・氏名　特任准教授　鈴木　亨

下記のとおり、西暦 2014 年 1 月 23 日に（修正の上承認・保留）として指示事項等のあった試験について以下のごとく回答します。

記

整理番号	P2013048-11Y
試験課題名	冠動脈カテーテル治療後再狭窄の新規バイオマーカー（BNP 断片比）の探索的臨床試験
指示事項	試験実施計画書および説明文書について、当日提出された修正案のとおり修正すること。
回答 （同意説明文書等の訂正を行った場合は、訂正後のものを添付する）	試験実施計画書および説明文書について、当日提出した修正案のとおり修正した。訂正後の試験実施計画書および説明文書を添付する。
備　考	説明文書・同意文書第 2 版（2011 年 1 月 29 日）
確認欄 確認日、確認印	臨床研究支援センター　荒川義弘 西暦 2014 年 2 月 4 日
	臨床試験審査委員会委員長* 西暦 2014 年 2 月 5 日

上記の試験において、以上の修正が適切に修正されていることを確認いたしました。
西暦 2014 年 02 月 05 日

病院長

*条件付き承認時の回答の場合に使用
未承認薬等の臨床使用の場合は、「（臨床）試験」とあるのを「（臨床）使用」と適宜読み替えるものとする。

(2012.5.30)

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名・刊号・ページ・出版年
Fujimoto H, Suzuki T, Aizawa K, Sawaki D, Ishida J, Komuro I, Nagai R	Processed B-type natriuretic peptide is a biomarker of post-interventi restenosis in ischemic heart disease.	Clin Chem, 2013, 59(9): 1330-7,
Suzuki T, Sawaki D.	Targeting Transforming Growth Factor-ß Signaling in Aortopathies in Marfan Syndrome.	*Circ J.* 2013, 25; 77(4): 898-9.
Bossone E, Suzuki T, Eagle KA, Weinsaft JW.	Diagnosis of Acute Aortic Syndromes - Imaging and Beyond.	*Herz.* 2013, 38(3): 269-76.
Suzuki T, Bossone E, Sawaki D, Jánosi RA, Erbel R, Eagle K, Nagai R.	Biomarkers of aortic diseases.	*Am Heart J,* 2013, 165: 15-25.

Clinical Chemistry 59:9
1330–1337 (2013)

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

Hirotaka Fujimoto,[1,2†] Toru Suzuki,[1,3†*] Kenichi Aizawa,[1†] Daigo Sawaki,[1,3] Junichi Ishida,[1] Jiro Ando,[1] Hideo Fujita,[1] Issei Komuro,[1] and Ryozo Nagai[1]

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-

[1] Department of Cardiovascular Medicine and [3] Department of Ubiquitous Preventive Medicine, The University of Tokyo, Tokyo, Japan; [2] Life Science Research Center, Technology Research Laboratory, Shimadzu Corp., Kyoto, Japan.
[†] H. Fujimoto, T. Suzuki, and K. Aizawa contributed equally to this work.
* Address correspondence to this author at: Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Fax +81-3-5800-9847; e-mail: torusuzu-tky@umin.ac.jp.

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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

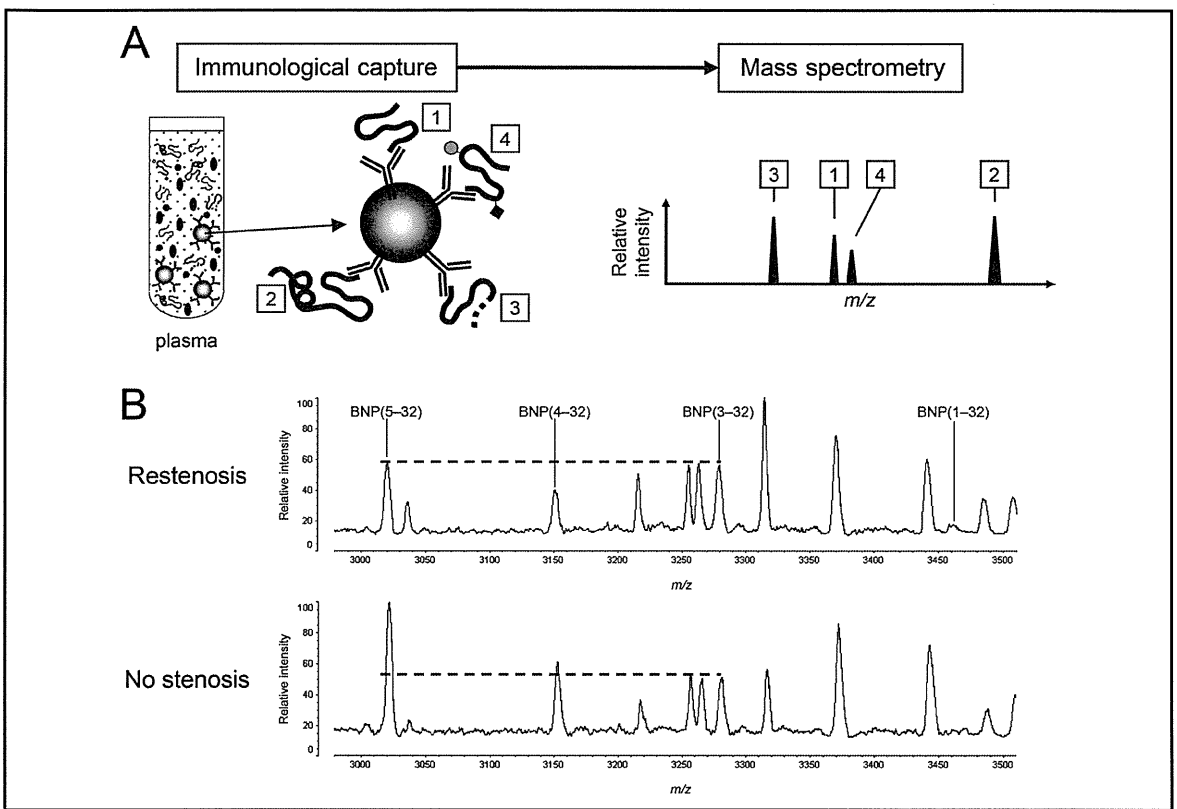

Fig. 1. Mass spectrometry–based immunoassay.

(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-

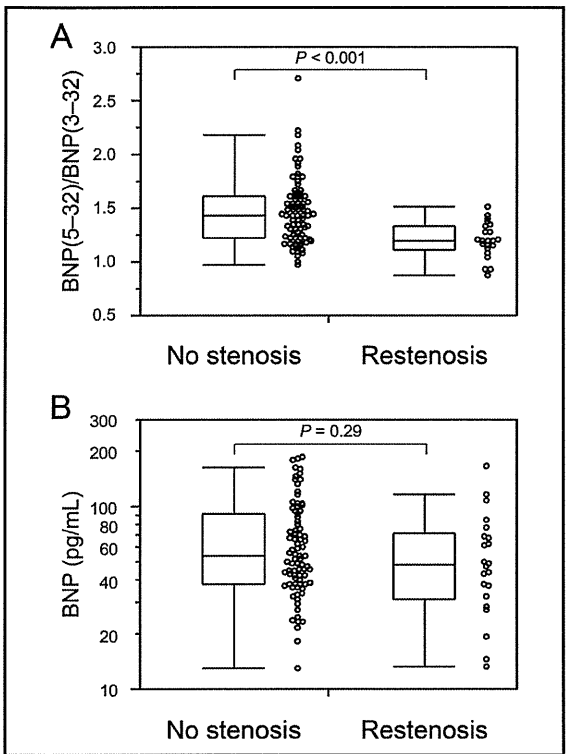

Fig. 2. BNP(5–32)/BNP(3–32) ratio and total concentrations of BNP in restenosis.

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.

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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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