厚生労働科学研究費補助金

医療機器開発推進研究事業

心臓カテーテル検査の低侵襲性代替診断法の開発
－被曝量低減化に向けた革新的体外診断薬の開発－

平成２４年度　総括研究報告書

研究代表者　鈴木　亨

平成２５（２０１５）年　５月

別紙１

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Ⅰ．総括研究終了報告

別紙３

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研究代表者　鈴木　亨

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研究要旨

本研究では心臓カテーテル検査に代替する低侵襲性診断法の開発を目指す。心臓カテーテル検査の被曝は医療被曝の中で最大のものの一つであり、その低減化が求められている。冠動脈疾患の主な治療法であるカテーテル治療では約1割～3割程度の症例で再狭窄（約半年後に病変が再度進行ないし治療に対する反応として狭窄する現象）が発症する問題ないし限界がある。再狭窄の予防を目的とした治療デバイスの改良が重ねられ、高度な薬剤溶出ステントが使われている現在においても、未だに治療後の患者の約1割に再狭窄が生じる。再狭窄を予知する方法がないため、治療半年後に再度心臓カテーテル検査を実施し再狭窄の有無を評価する必要があるが、X線照射を必要とする検査であり、安全性また医療費の面からも血液検査等の代替法の開発は急務であった。我が国における心臓カテーテル治療半年後に行われる検査件数は年間約26.2万件（推定）である。カテーテル検査１回ごとの被曝量は約7.5mSvであり（他の放射線を用いた医療用検査と比較し数倍～数十倍）、約30万円の費用がかかる。代替検査法の開発により被曝量軽減（患者、術者ともに）また医療費削減も可能となる。

我々は心臓カテーテル治療後の再狭窄を反映する世界で初めてのバイオマーカーを最近開発した（特許も申請済み）。心臓カテーテル検査に代替する方法としてこのバイオマーカーを用いることで被曝量の低減化を図る。すでに実施した160例での初期検討で再狭窄の検出と予知において特異度100%と抜群の性能を示した。本法は、心血管病態時に特異的に発現する蛋白質のナトリウム利尿ペプチド(BNP)から慢性心筋虚血時に生じる特異なプロセシング産物を質量分析計で測定する方法である。本研究中に検査法の有用性の確立を目指す。研究初年度（平成23年度）は計画通り分析を行い、再狭窄の除外診断法としての性能（検出能ならびに予測能）を検証した（横断258例、縦断64例）。二年目も計画通りに症例を重ね（横断306例、縦断99例）、本診断法の有用性を検証した。最終年度は先進医療に向けた前処理、検出法等の最適化を目指した解析を実施する。

研究終了後には先進医療の申請、薬事承認申請を目指す。被曝量の低減化、低侵襲の革新的診断機器を開発する研究、低侵襲かつ患者の視点から苦痛の少ない革新的治療機器を開発する研究となるものと期待している。

A．研究目的

心臓カテーテル検査の被曝は医療被曝の中で最大のものの一つであり、その低減化が求められている。昨今の心臓カテーテル法を用いた検査・治療法の普及にともない、冠動脈疾患に対する高度な治療が普及した一方、患者の放射線皮膚障害の増加とともに、白内障や甲状腺機能低下症などの術者における健康障害も懸念される。このため、X 線を使用しない冠動脈疾患の診断法の開発が必要とされている。

本研究では心臓カテーテル検査に代替する低侵襲性診断法の開発を目指す。具体的には、心臓カテーテル治療実施半年後に再狭窄の検出を目的に行われる心臓カテーテル検査に代替する方法として我々が開発した診断バイオマーカーを用いる計画である。冠動脈疾患に対するカテーテル治療の主な問題点ないし限界は、約1割〜3割の症例で再狭窄（約半年後に病変が再度進行ないし治療に対する反応として狭窄する現象）が発症することがあげられる。現在、再狭窄を予知する方法がないため、治療半年後に再度心臓カテーテル検査を実施し、再狭窄の有無を評価する必要がある。医療被曝の低減、安全性また医療費の面からも血液検査等の非観血的な診断法の開発は急務である。

我々は心臓カテーテル治療後の再狭窄を反映する世界で初めてのバイオマーカーを最近開発した。心臓の慢性虚血時にみられるBNPの特異なプロセシング産物に着目し、再狭窄を示したあるいは将来的に示す症例では、このペプチドが減ることを世界で初めて見いだした。160例を用いた初期検討では心臓カテーテル治療後の再狭窄の検出また予知において本法は特異度100%と抜群の性能を示した。特許も申請済みである。本研究期間中に検査法の有用性の確立を目指す。具体的には、横断的・縦断的に症例数を増やし、3年間で1,000例（横断例800例、縦断例200例）を目標とすることで代替法としての診断的な意義ならびに臨床的な有用性を確立する。

研究終了後には先進医療の申請、薬事承認申請を目指す。被曝量の低減化、低侵襲の革新的診断機器を開発する研究、低侵襲かつ患者の視点から苦痛の少ない革新的治療機器を開発する研究となるものと期待している。

B．研究方法

我々が開発した、心臓カテーテル検査の代替法となる本バイオマーカーは、心血管病態時に特異的に発現する蛋白質であるナトリウム利尿ペプチド(BNP)から、慢性虚血時に生じる特異なプロセシング産物を質量分析計で測定することを測定原理とする（図1）。BNPは心筋虚血、心不全等の病態において心臓で特異的に生成され、また血中に放出されることにより腎臓や血管をはじめ

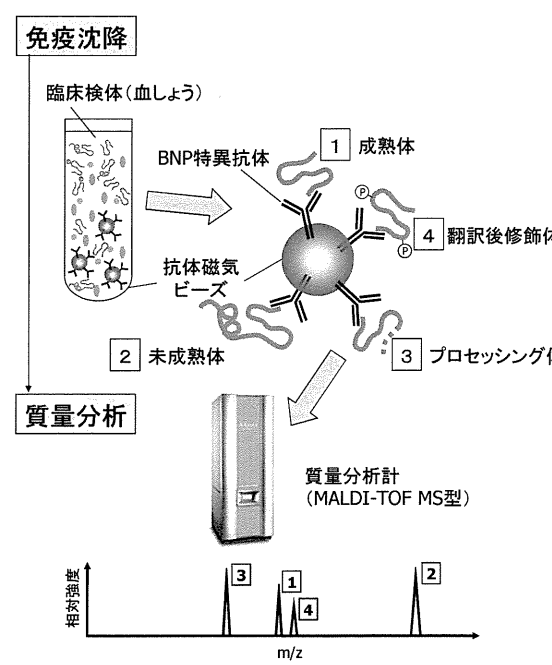


図1. 測定原理

とする臓器に作用し、血管拡張ないし利尿作用等の効果を通して血行動態の安定性に寄与する、我が国で発見された心臓由来の生理活性ホルモンである。血中BNP値の測定を通した心不全の診断は10年以上前から臨床導入され、現在は世界中でもっとも広く使われている心臓血管領域の診断バイオマーカーのひとつである。先述したように、我々は慢性虚血時にみられるBNPの特異なプロセシング産物に着目し、再狭窄を示したあるいは将来的に示す症例では、このペプチドが減ることを見いだした。160例での初期検討において、心臓カテーテル治療後の再狭窄の検出また予知において本法は特異度100%と抜群の性能を示した。特許も申請済みである。

具体的な計画として、心臓カテーテル検査の代替法としての診断的意義また臨床的有用性を確立するために、前向きコホートによる検証とともに、横断解析を拡げる。実際、研究初年度から二年目にかけて分析検体数を増加し、病態変遷との関係をさらに検討した結果、この診断法の性能は

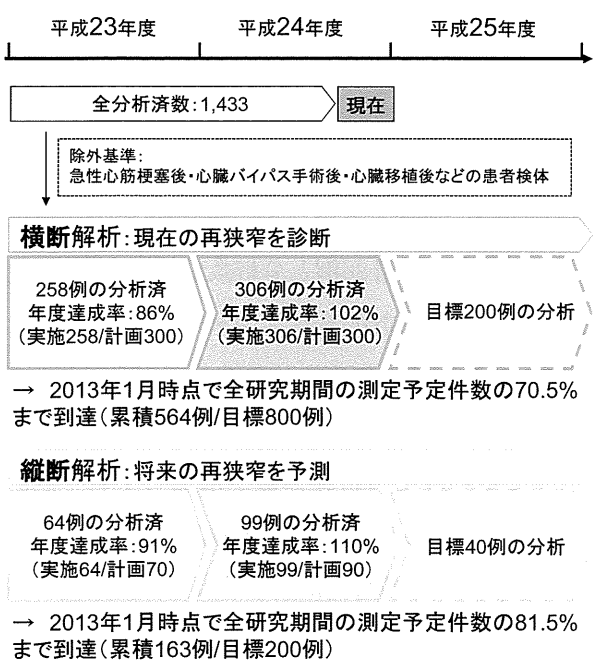


図2 分析の進捗状況

維持されていることが判明した。今後、臨床導入への第一歩として再狭窄のバイオマーカーとしての有用性を確立するとともに、先進医療申請を目指す。代替法としての臨床的な有用性が示されれば、被曝量の低減化を図ることにより心臓カテーテル治療の安全性を高めるとともに、再狭窄リスクの評価を通して治療法選択にも寄与すると期待している。

すでに初期検討にて、130例程度の横断研究ならびに30例程度の前向き検討を実施したが、本研究では、さらに800例程度の横断測定を行い、また200例程度の縦断検討を行う。研究期間中に計1,000例を測定する。研究に必要な血液は医学部附属病院循環器内科を中心に心臓カテーテル検査症例の検体を入手する。研究初年度(平成23年度)は計画通り分析を行い、再狭窄の除外診断法としての性能(検出能ならびに予測能)を検証した(横断258例、縦断64例)。研究二年も計画通りに症例を重ねた(横断306例、縦断99例)、最終年度は先進医療に向けた前処理、検出法等の最適化を目指した解析を実施する。

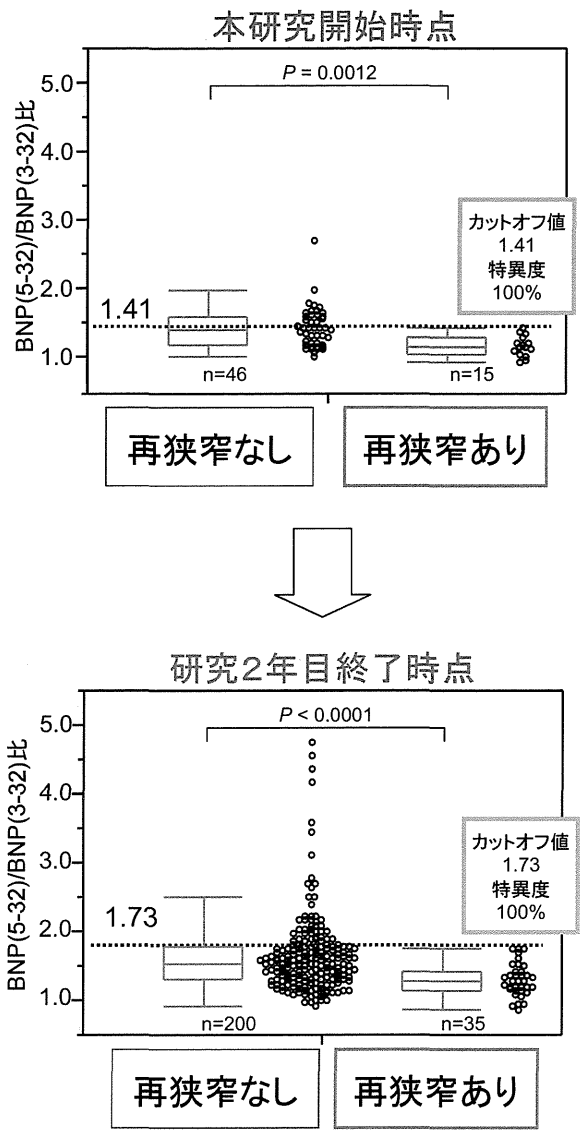
(倫理面への配慮)

本研究(東京大学医学部附属病院循環器内科に循環器疾患で入院した症例および同院検診部を受検した健常例に対するプロテオーム解析による新規診断法の開発)はすでに当施設の倫理委員会の承認(平成21年6月19日承認、承認番号448(2))を得ており、当院の倫理規定に従って遂行した。

C. 研究結果

研究二年目(平成24年度)は計画通り、さらに検体分析を行い、初年度に得られた再狭窄の除外

診断法としての性能（検出能ならびに予測能）を検証した。図2に現在までの分析進捗状況を示す。研究初年度（平成23年度）の分析数が横断258例、縦断64例であった。本年度（研究二年目）の分析



	本研究開始時点	研究2年目終了時点
狭窄無し	46	200
再狭窄	15	35
P値	0.0012	< 0.0001
AUC	0.782	0.730
カットオフ値	1.41	1.73

図3 横断解析:カテーテル治療後の再狭窄診断

数は横断306例、縦断99例であった。

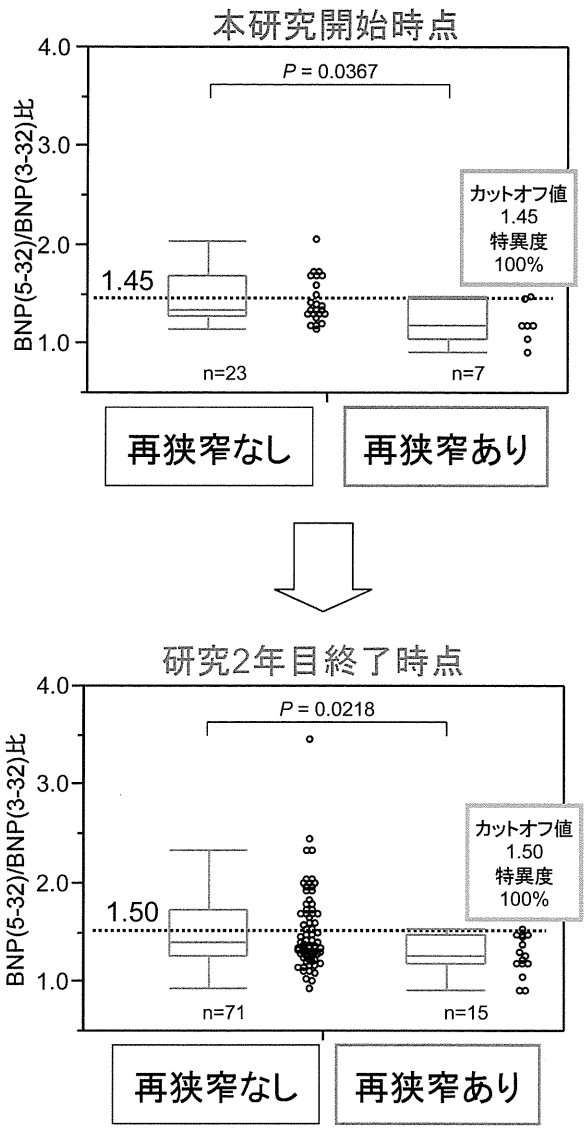
具体的な成果として、再狭窄の検出能を検討する横断解析、すなわち心臓カテーテル治療後約半年経過時点での再狭窄の有無を診断する診断の結果は以下の通りである。研究二年目（平成24年度）に実施済みの分析306例のうち、心不全・腎不全等の除外規定に該当する症例を除き、統計分析基準を満たした分析例数は154例であった。具体的な除外規定は以下の通りである。腎機能が低下している症例（クレアチニン値 2.0 mg/dL以上）、心不全症例（BNP値200 pg/mL以上）、新規病変を生じていた症例、分析系の感度限界以下のためにシグナル検出不可であった検体、について統計解析から除外した。この統計基準を満たした分析例をこれまでの横断解析の分析例に追加し、統計解析を行った（図3）。その結果、再狭窄の検出において非狭窄群と狭窄群の統計的有意差(p値)は前年度の0.0001未満を維持した。また、診断性能を示す受信者操作特性曲線(ROC)の曲線下面積(AUC)値は0.730と、ほぼ前年度と同じ水準を維持していた。このことから、本年度に分析数を増加させても本診断法の診断能力が維持されていることが確認された。

一方、縦断的解析、すなわち心臓カテーテル治療前に治療後の再狭窄を予知する診断は、前年度の21例から計86例と分析数が増加したが、p値は0.0218と統計的有意水準を維持した（図4）。また、AUC値は前年度と比較して0.689とやや小さくなったが、ほぼ同じ水準を維持した。このように本年度に分析検体数が増加しても予測診断能力に変わりの無いことが確認された。

これまでに、横断例に関しては全研究期間の測定予定件数の70.5%まで到達し（累積564例/目標800例）、縦断例に関しては81.5%まで到達した（累

積163例/目標200例）（図2）。現在まで、分析数を増やしてもこれまでの診断性能を維持していることがわかった。

以上のように、現状では当初の予定通りの計画が進行しており、予想通りの成果も得られている。



	本研究開始時点	研究2年目終了時点
狭窄無し	23	71
再狭窄	7	15
P値	0.0367	0.0218
AUC	0.767	0.689
カットオフ値	1.45	1.50

図4. 縦断解析:カテーテル治療後の再狭窄予測

最終年度は、さらに分析例数を重ねつつ、本診断手法の先進医療への申請を目指す。すなわち、先進医療に向けた前処理、検出法等の最適化を目指した解析を実施するとともに、プロトコルの確立を行う。

将来的には体外診断薬として広く一般に提供できる体制を整えるため、研究終了後には先進医療の申請、薬事承認申請を目指す。再狭窄リスクを本法で評価することで、被曝量の低減化を図ることにより心臓カテーテル治療の安全性を高めると同時に、再狭窄リスクの評価を通して、治療法選択への寄与も期待している。

なお、心臓カテーテル法を用いた検査・治療法は近年普及し、今日では年間46.0万人の患者がカテーテル検査を、26.2万人の患者がカテーテル治療を受けている（2008年循環器疾患診療実態調査報告書による。また、この調査の回答率は国内全施設の約40%のため、実際にはこれを相当数上回る）。カテーテル治療後の再狭窄に懸念に対して、治療半年後に再度心臓カテーテル検査が実施されている。すなわち、26.2万回の確認目的の心臓カテーテル検査が行われていると推定される。また、心臓カテーテル検査の1回あたりの被曝量は約7.5mSv、またカテーテル治療で15mSvであり、現在、医療検査に伴う被曝の中で最大のものの一つである。例えば、CT等の診断目的の他のX線検査（0.5～1.0mSv、放射線技師会雑誌No47 10号）と比較し、被曝量は数倍～数十倍大きい。カテーテル検査の費用は１件約30万円であるが、わが国全体で786億円の医療費の負担ともなっている。本検査の費用を仮に１件5,000円と試算しても97%減となり、検査費用は23億円に抑えられ、その

差は実に763億円超となる。

心臓カテーテル治療後に実施される再狭窄を対象に行われる確認目的の心臓カテーテル検査に代替する血液検査を開発することで、上記のように被曝量軽減、医療費軽減の両者の視点から非常に大きい効果が期待できる。

D．健康危険情報

該当無し。

E．研究発表

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F．知的財産権の出願・登録状況

1. 特許取得

1. 鈴木亨、日本電気株式会社 宮崎賢司 他、積水メディカル株式会社：心疾患診断マーカー 特許出願2011-175982（出願日２０１１年８月１１日）出願人：東京大学、ＮＥＣ、積水メディカル

2. 鈴木亨、藤本宏隆：血液試料を用いて心筋虚血状態を評価する方法 国際出願PCT/JP2008/65444（２００８年８月２８日）出願人：国立大学法人東京大学、株式会社

島津製作所

2. 実用新案登録

該当無し。

3. その他

該当無し。

II. 研究成果の刊行に関する一覧表

別紙５

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	巻号	ページ	出版年
Fujimoto H, Suzukonali 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	未定	未定	2013(in press)
相澤 健一，鈴木 亨	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(3.動脈硬化のバイオマーカーの臨床的意義,〈特集〉第76回日本循環器学会学術集会)	循環器専門医 ： 日本循環器学会専門医誌	20(2)	237-244	2012
相澤 健一，鈴木 亨	バイオマーカーの探索・発見・同定の試（５．今後のバイオマーカーの展望，特集：循環器病のバイオマーカー企画・構成／井上晃男）	Heart View	16(12)	306-310	2012

III. 研究成果の刊行物・別刷

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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 synthesized as a propeptide, preproBNP(1–134), that under-

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[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.

goes 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 EDTAate-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 stepwise variable selection was performed starting with

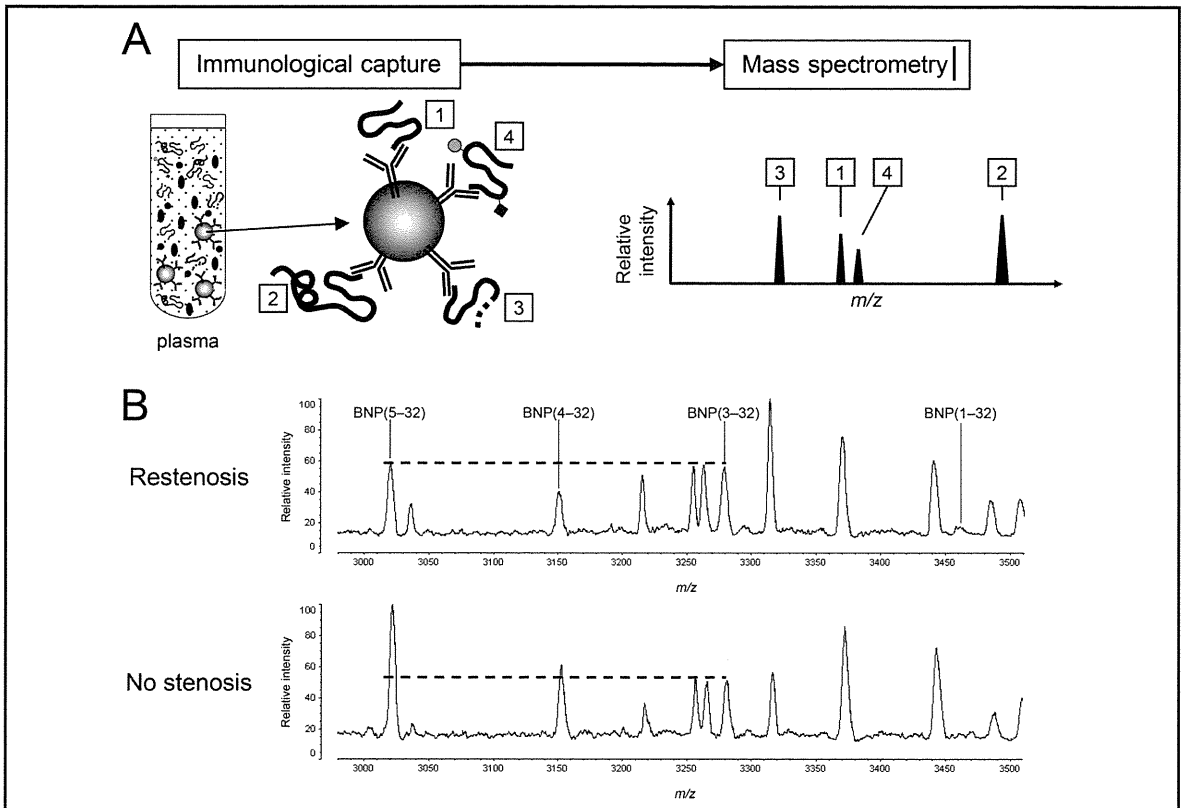

Fig. 1. Mass spectrometry–based immunoassay.

(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.

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 to serve as an internal control to quantify concentra-

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.

tions 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 concentrations of BNP and BNP(5–32)/BNP(3–32) ratio in

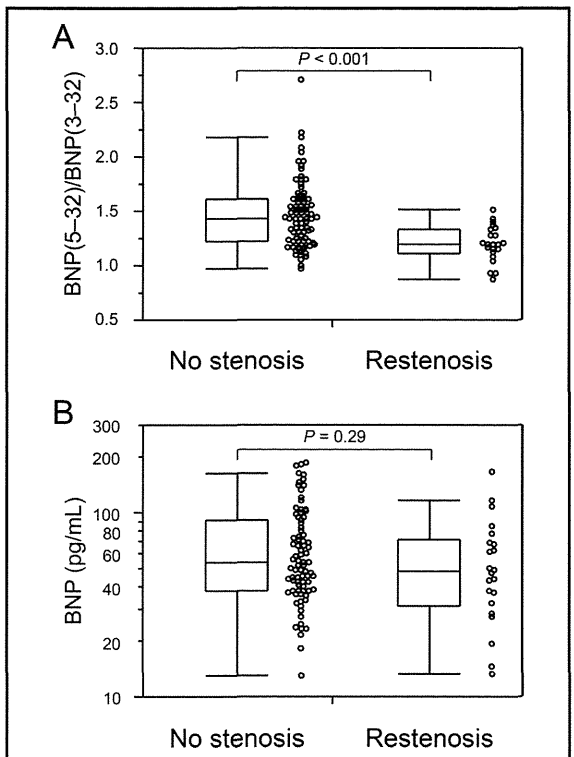

Fig. 2. BNP(5–32)/BNP(3–32) ratio and total concentrations of BNP in restenosis. Association between restenosis diagnosed by CAG and BNP(5–32)/BNP(3–32) ratio (A) and total concentrations of BNP in representative patients with and without restenosis (B) in the cross-sectional study.

No stenosis, n = 83; restenosis, n = 22. Boxes represent IQR, and the horizontal line in each box represents the median.

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-