A Combination Assay with B-type Natriuretic Peptide (BNP) and High Sensitive Troponin I (hsTnI) for the Detection of Potential Cardiovascular Diseases

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Satoshi SUGAWA

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Satoshi SUGAWA

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Abbreviations

ACS	Acute coronary syndrome
ALB	Albumin
ALT	Alanine aminotransferase
ANP	Atrial natriuretic peptide
AST	Aspartate aminotransferase
BMI	Body mass index
BNP	B-type natriuretic peptide
BPM	Beats per minute
BUN	Blood urea nitrogen
CKD	Chronic kidney disease
CLSI	Clinical and Laboratory Standards Institute
CNP	C-type natriuretic peptide
CNS	Central nernous system
CRE	Creatinine
cTn	Cardiac troponin
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CTR	Cardiothoracic ratio
CV	Coefficient of variation
CysC	Cystatin C
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
ET-1	Endothelin-1
FBG	Fasting blood glucose
FRS	Framingham risk score
GDF-15	Growth-differentiation factor 15
GGT	Gamma glutamyl transferase
HbA1c	Hemoglobin A1c
HDL-C	High-density lipoprotein cholesterol
HE4	Human epididymis protein 4
hFABP	Heart-type fatty acid-binding protein
HFmrEF	Heart failure with mid-range ejection fraction
HFpEF	Heart failure with preserved ejection fraction

HFrEF	Heart failure with reduced ejection fraction
HR	Heart rate
Ht	Hematocrit
ICAM	Intracellular adhesion molecule
IHD	Ischemic heart disease
LDL-C	Low-density lipoprotein cholesterol
LoB	Limit of blank
LoD	Limit of detection
LoQ	Limit of quantitation
LVEF	Left ventricular ejection fraction
MR-proADM	Mid-regional pro-adrenomedullin
NGAL	Neutrophil gelatinase-associated lipocalin
NPPB	Natriuretic peptide precursor B
NRG-1	Neuregulin 1
NT-proBNP	N-terminal pro B-type natriuretic peptide
PLT	Platelet count
RAAS	Renin-angiotensin-aldosterone system
RBC	Red blood cell count
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Structural equation modeling
sST2	Soluble suppression of tumorigenicity 2
TG	Trilyceride
UA	Uric acid
ULN	Upper limit of normal
VC	Vital capacity
WBC	White blood cell count
WC	Waist circumference

Chapter 1. Background of This Study

1. Cardiovascular Disease in an Aging Country Like Japan

Cardiovascular disease is the second highest cause of death in Japan and the incidence is increasing year by year. Ratio of population over 65 of age has exceeded 27% of the total population as of 2016 which is at the top in the world and is expected to approach 40% in 50 years [1]. The national medical expenditure in Japan which already exceeds 40 trillion yen is speculated to exceed 50 trillion yen in 2030 [2]. It may well be construed, therefore, as that Japan is the front runner among aging countries in the world so that Japan is expected to show to the following countries solutions or directions for economically and culturally sustainable development. One of the solutions would be to find diseases at their early stage and intervene with them before they develop into severe status so that people could live healthy life longer with the less medical expenditure.

According to statistics, more than 70% of the cause of the sudden death is attributed to cardiac diseases by which 30,000 to 40,000 people die every year in Japan [3]. The number of patients with heart failure in Japan is estimated to be approximately one million currently, which is 0.8% of the total population and the

number is estimated to reach 1.3 million by 2030 [4]. It is pointed out that the patients with heart failure, in many cases, are diagnosed as having heart failure only after their disease status have been advanced [5]. Under this situation, I believe, to develop an efficient method to screen the general population for potential cardiovascular diseases, such as a blood test for detection of biomarkers, would be extremely important for the welfare of the aging society like Japan.

2. Overview of Groups of Cardiovascular Diseases

In terms of the number of deaths by cardiovascular disease groups, heart failure is at the top (37%) among cardiovascular disease groups followed by myocardial infarction (18%), other ischemic cardiovascular diseases (17%), and arrhythmia (16%) as shown in Fig. 1 [6]. Followings are brief descriptions on the above cardiovascular disease groups.

1) Heart Failure

Heart failure is a general term to define cardiovascular diseases that show reduced heart function by increased myocardial mass and left ventricular hypertrophy as a consequence of long-term exposure to elevated mechanical

stress by hypertension or some other factors. This disease group is categorized further into three sub-groups according to the function of left ventricle ejection fraction (LVEF); heart failure with preserved ejection fraction (HFpEF), heart failure with mid-range ejection fraction (HFmrEF) and heart failure with reduced ejection fraction (HFrEF). The LVEF's of HFpEF, HFmrEF and HFrEF are defined as equal to or more than 50%, equal to or more than 40% and less than 50%, less than 40%, respectively [7].

2) Ischemic Heart Disease

Ischemic cardiovascular disease is a general term to define cardiovascular diseases caused by disrupted blood stream at coronary arteries such as by plaque rupture, embolism or dissection. Myocardial infarction belongs to this group which is defined as a cardiovascular disease with blocked blood stream at coronary arteries by plaque rupture and the thrombosis [8].

3) Arrhythmia

Arrhythmia is a general term to define cardiovascular diseases with irregular heart beat which include tachycardia (>100 beats per minute, or bpm),

bradycardia (<60 bpm) and a one with aberrant pulses [9].

4) Other Cardiovascular Diseases [10, 11]

Other than the cardiovascular diseases described above, there are pericardial heart disease, valvular heart disease, cardiomyopathies, congenital cardiovascular diseases, etc.

Pericardial cardiovascular disease is a general term to define diseases with defective pericardium, which includes acute pericarditis, pericardial effusion, cardiac tamponade, and constrictive pericarditis.

Valvular heart disease is a general term to define cardiovascular diseases caused by the disorder at heart valves such as pulmonary valve, aortic valve, mitral valve, or tricuspid valve.

Cardiomyopathy is a general term to define cardiovascular diseases with disorder at the heart muscle. Although primary cardiomyopathies are generally confined to the disorder at the heart muscle, secondary cardiomyopathies not only show disorders at myocardium but also at systemic multi-organs.

Congenital cardiovascular disease is a general term to define cardiovascular diseases caused by inherited genetic and structural defects. There are six major

categories in the congenital heart defects; arterial septal defects, congenitally corrected transposition of the great vessels, tetraology of Fallot, transposition of the great arteritis, Ebstein anomaly and Fontan circulation.

3. Risk Factors for Cardiovascular Diseases

Since 1960's, studies from Framingham and other epidemiological cohorts contributed in establishing a concept of 'risk factors' for the development of cardiovascular diseases, which include hypertension, dyslipidemia, diabetes mellitus and smoking habit [12]. Basing on this concept, many researchers attempted to create multivariable algorithms for risk scores of cardiovascular diseases by applying the data obtained in the Framingham Heart Study. Among these efforts, D'Agostino *et al.* proposed a multivariable risk assessment tool that enables physicians to identify high-risk population using measurements readily available at clinics (e.g. sex, age, high-density lipoprotein cholesterol (HDL-C), systolic blood pressure (SBP), smoking habit, diabetes, etc.), where the risk score derived from this algorithm is defined as the probability of 10-year cardiovascular incidence [13].

In Chapter 2, I assessed the associations between selected biomarkers and risk

factors mentioned above as well as the association with the Framingham Risk Score (FRS) calculated by the D'Agostino's algorithm.

4. Selection of Suitable Biomarkers for the Screening of Cardiovascular Diseases [14]

1) Biomarkers' Contribution in Preventing the Development of Cardiovascular Diseases

As described in the previous section, risk factors such as hypertension, dyslipidemia, diabetes mellitus, chronic kidney disease or smoking habit have been proven to have strong association with the development of cardiovascular diseases (Fig. 2). Because it usually takes many years for people having these risk factors to develop serious cardiovascular diseases, it is difficult to decide at which time point doctors need to intervene (e.g. to do operational treatment) in addition to instructing the patients to improve their lifestyle (e.g. to go on a diet, to do more exercise, etc.). In this sense, desirable cardiac biomarkers would be those which show direct sign that the heart is starting to go wrong so that the doctors would be able to know when to intervene.

2) Overview of Candidate Biomarkers Related with Cardiovascular Diseases

To overview established and emerging biomarkers that are related with cardiovascular diseases, I listed up representing eleven biomarkers in Table 1 and Fig. 3. More specifically, I summarized the physico-chemical property of the molecule (e.g. protein, peptide, lectin, etc.), the function (e.g. hormone, enzyme, structural protein, receptor, etc.), and the suggested indication for its clinical usage in Table 1 while I categorized these biomarkers in the context of physiological responses such as myocardial injury, myocardial stress, oxidative stress, inflammation and so on in Fig. 3. As can be seen, some biomarkers appear in multiple categories owing to the nature of the biomarkers (e.g. sST2, Pentraxin 3).

3) Specifications of Desirable Biomarkers from Analytical Aspect

From the aspect of analytical requirements, desirable biomarkers are expected to have good performances in sensitivity, specificity, reproducibility, accessibility, cost-effectiveness. From the analytical aspect, sensitivity is defined as the

minimal concentration that the assay can detect (for the detail, see “Three Definitions of Sensitivity; LoB, LoD and LoQ” in Section 6 in this chapter), specificity is defined as the ability of the assay to discern the target molecule from others, reproducibility is defined as the ability of the assay to quantitate within pre-defined fluctuation range, accessibility means how easily the assay can be accessed including the factors of reagent supply and automation, cost-effectiveness means how effectively the assay cost can be managed in clinical laboratories (e.g. less labor intensive work).

4) Specifications of Desirable Biomarkers from Clinical Aspect

From the aspect of clinical requirements, desirable biomarkers are expected to have good specificity to the disease of interest and show good performances in the diagnosis, assessment of severity, monitoring of the treatment and assessment of prognosis. Specificity in terms of clinical aspect means specific association of the target molecule with the target disease. Therefore, if a target molecule is not only released from heart muscle but also from skeletal muscle, for example, the specificity of this biomarker could be considered as “compromised” in terms of the specificity for heart disease.

5) Selection of Biomarkers for the Screening of Cardiovascular Diseases

Because there are variations in analytical performances by assays from different manufacturers, I focused on the clinical characteristics of the target biomarker for the selection here. As shown in Table 2, I assessed 13 biomarkers from the clinical aspects of specificity, utilities for diagnosis, severity, monitoring and prognosis as mentioned above. For the assessment, I used three categories; satisfactory (○), not decided or compromised (△), and not suitable (×) and categorized each by previously reported evidences (Table 2). Specification was categorized as “satisfactory” when it is released only from the target organ and not affected by other diseases. For example, the specificity of cardiac troponin T (cTnT) was categorized as “not decided or compromised” because it is released not only from the heart but also from the skeletal muscle in patients with renal failure [15] or skeletal muscle disease [16]. Similarly, NT-proBNP was categorized as “not decided or compromised” because its level is significantly affected by renal failure [17].

As the result of the assessment, B-type natriuretic peptide (BNP) and cardiac

troponin I (cTnI) showed the best clinical performances among the 13 biomarkers as shown in Table 2. Because BNP is released by hemodynamic stress on the heart especially when the heart wall is thickened, or at the state of hypertrophy, while cTnI is released by myocardial injuries such as when there is necrosis on the myocardium, I thought these two biomarkers would cover broader sub-population at risks with clinical and sub-clinical cardiovascular diseases in the general population as illustrated in Fig. 4. I, therefore, assumed that the combination assay of BNP and cTnI would be a better screening method to cover broader sub-population than single assays and decided to investigate the validity of the combination assay in this study.

5. Objectives of This Study

Because of the reasons illustrated above, I selected the two biomarkers, BNP and cTnI, and assessed the validity of the combination assay of the two biomarkers in the general population. More specifically, the objectives of this study are as follows;

1) To assess whether BNP or cTnI shows association with the risk factors for cardiovascular diseases as mentioned earlier,
2) To assess whether BNP and cTnI detect different populations and different types

of (sub-clinical) cardiovascular diseases, and

3) To assess whether there is any positive or inverted association between BNP and cTnI.

6. Statistical Analyses

1) Shapiro-Wilk Test

Shapiro-Wilk test is one of the most popular statistical methods to assess whether a distribution of a given numerical group conforms with Gaussian distribution or not [18]. Gaussian distribution is called “parametric,” whereas non-Gaussian distribution is called “non-parametric”.

2) Student’s T-test [19, 20]

Student’s t-test is a statistical method to assess whether the difference between two parametric distributions is significant. When you compare two populations where samples of the two populations can be paired (e.g. weights before and after exercise of the same person), “paired t-test” is used, while you compare two populations with unpaired samples, “unpaired t-test” is used.

3) Wilcoxon's Test [21]

Wilcoxon's test is a statistical method to assess whether the difference between two non-parametric distributions is significant.

4) Linear Regression Analyses [22]

Linear regression analysis is a statistical method to assess whether a given variable is significantly associated with a variable of interest. For example, association of age (shown in the X-axis) against cTnI (shown in the Y-axis) is analyzed by examining the linear relationship between the two variables as illustrated in Fig. 5. "Univariable linear regression" analyzes the linear relationship between the two variables as mentioned above by the equation

$$Y = a \times X + b$$

where "a" is the slope and "b" is the y-intersect of the line. On the other hand, "multivariable regression" analyzes the linear relationship between a variable of interest (designated as Y) and multiple independent variables (designated as X_i) by the following equation

$$Y = a_1 \times X_1 + a_2 \times X_2 + \ldots + a_n \times X_n + b.$$

This model permits the computation of a regression coefficient a_i for each independent variable X_i.

5) Exclusion of Outlying Test Results by Dixon's Method [23]

Dixon's method is a statistical method to assess whether outlying data should be excluded from further statistical analyses. The formula is defined as D/R, where D is a difference between the largest (or smallest) value and the second largest (or smallest) value, and R is the range of all the data. Reed *et al.* suggests that one-third as a practical cut-off.

6) Three Definitions of Sensitivity; LoB, LoD and LoQ [24, 25]

As there are several ways to define sensitivity, limit of blank (LoB), limit of detection (LoD) and limit of quantitation (LoQ) are standard methods to define the sensitivity proposed by Clinical and Laboratory Standards Institute (CLSI) as illustrated in the guideline EP17-A, "Protocols for Determination of Limits of Detection and Limits of Quantitation".

LoB is obtained by measuring replicates of a blank sample and is calculated from the following formula with the mean and the standard deviation (SD);

$$LoB = mean_{blank} + 1.645 \times (SD_{blank})$$ [Fig. 6]

where SD_{blank} signifies a standard deviation of the blank sample,

LoD is estimated by the following formula;

$$LoD = LoB + 1.645 \times (SD_{low\ concentration\ sample})$$ [Fig. 6]

where $SD_{low\ concentration\ sample}$ signifies a standard deviation of the low concentration sample and 1.645 signifies one-sided 95% confidence interval.

LoQ is defined as the lowest concentration at which the sum of functional sensitivity and bias is within certain predefined allowance range where the functional sensitivity is defined as the lowest concentration that shows CV% within predefined allowance range, and the bias is defined as the percentage of deviation from the linearity. For the assessment of LoQ in the clinical practice, 30% is one of the frequently used allowance ranges [Fig. 7].

7) Covariance Structure Analysis [26]

Covariance Structure Analysis, or Structural Equation Modeling, is a general statistical modeling technique which could be understood as a combination of factor analysis and multiple regression analysis. The covariance structure is a structure where the relationships between the variables are connected by path

coefficients between them (Fig. 8). By constructing the covariance structure model, or path model, you would be able to assess the strength and the direction (positive or inversed) of the relationships between the variables.

Table 1: Summary of Cardiac Biomarkers

	Pathophysiology and Proposed Clinical Utility
Copeptin	Copeptin is a C-terminus portion of pro-vasopressin, which is synthesized and secreted from the hypothalamus in response to change in plasma osmolarity and reduced cardiac output. In patients with heart failure, copeptin levels are highly predictive of mortality independent of other risk factors.
Cystatin C	Cystatin C (CysC) is a cysteine protease inhibitor produced at a constant rate by all nucleated cells of the body. Because several properties of CysC make it superior to creatinine as a measure of glomerular filtration rate (GFR), CysC is expected to be used as a marker for both acute kidney injury and chronic renal failure.
Endothelin 1	Endothelin-1 (ET-1) is a profibrotic hormone primarily secreted by endothelial cells, cardiomyocytes, smooth muscle cells and macrophages. Increased levels of ET-a have been noted in patients with chronic heart failure, but the prognostic value is not clear.
Galectin-3	Galectin-3 is a member of a family of soluble β -galactoside-binding lectins. Galectin-3 was initially identified as a mediator of tumor growth, but new data indicates a potential link between inflammation and fibrosis. In chronic heart failure, Galectin-3 promotes maladaptive cardiac remodeling by augmenting fibrosis and modulating immune response.
GDF-15	Growth-differentiation factor 15 (GDF-15) is a member of the transforming growth factor-β cytokine superfamily expressed in myocytes, smooth muscle cells, endothelial cells, adipocytes and atherosclerotic plaques. Studies have shown that elevated levels of GDF-15 are strongly associated with poor prognosis in patients with coronary diseases.
cTn	Cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are structural proteins expressed in myocardium. High sensitive assays are now able to detect not only ischemic necrosis of myocardium but also micro necrosis due to causes other than ischemia. cTn is currently used primarily for the diagnosis of acute coronary syndrome, but also is expected to be used as a prognostic marker as well.
MR-proADM	Mid-regional pro-adrenomedullin (MR-rpoADM) is a stable prohormone of adrenomedullin, which is a hormone having potent vasodilatory and natriuretic effects. Elevated levels of MR-proADM is strongly associated with poor prognosis of heart failure, but response to medical therapy is inconsistent among

	several studies.
Neuregulin 1	Neuregulin 1 (NRG-1) belongs to a family of growth factors that functions via receptor tyrosine kinases and has indispensable role in cardiac development and functioning. Levels of NRG-1 are independently associated with the severity of heart failure, but further studies are needed to establish its value as a prognostic marker.
NGAL	Neutrophil gelatinase-associated lipocalin (NGAL) is a small glycoprotein released from several types of cell, including epithelial cells, renal tubular cells, and hepatocytes, during inflammation or after injury. Measurement of urine NGAL is thought to have at least two potential uses; a marker to detect acute kidney injury and a predictor of adverse outcomes in chronic heart failure.
sST2	sST2 is a truncated soluble form of a member of interleukin-1 receptor family. Increased levels of sSTs could augment adverse remodeling and deemed to have an important prognostic value in patients with acute coronary disease or heart failure.
BNP	B-type natriuretic peptide (BNP) is a peptide primarily secreted from ventricular myocytes in response to hemodynamic stress. This peptide is extremely useful in determining whether heart failure is the cause of acute dyspnea, and in determining the severity and prognosis of heart failure.

Table 2: Specifications of Cardiac Biomarkers from Clinical Aspect

	Specificity	Diagnosis	Severity	Monitoring	Prognosis
Copeptin	×	△	○	△	○
Cystatin C	×	×	○	△	○
Endothelin 1	×	△	△	△	△
Galectin-3	×	△	○	×	△
GDF-15	×	△	○	△	○
cTnI	○	○	○	○	○
cTnT	△	○	○	○	○
MR-proADM	×	△	○	△	○
Neuregulin 1	△	△	△	△	△
NGAL	△	○	○	○	△
sST2	×	×	○	△	○
BNP	○	○	○	○	○
NT-proBNP	△	○	○	○	○

○: Satisfactory, △: Not Decided or Compromised, ×: Not Suitable

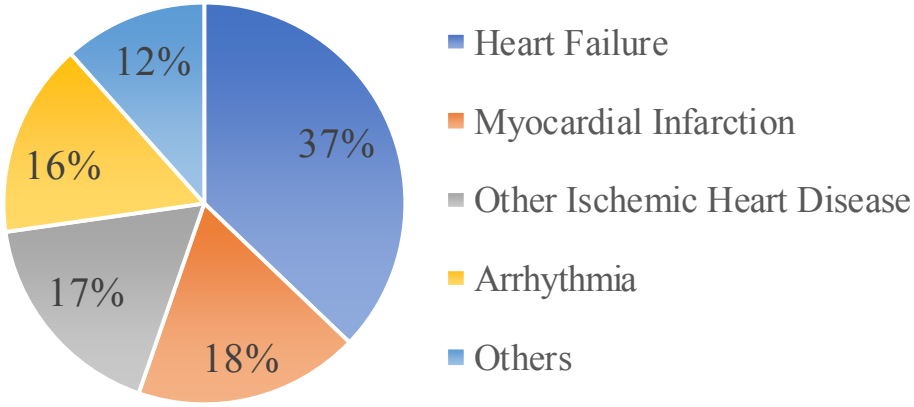

Fig. 1: Percentages of Cardiovascular Deaths by Cardiovascular Disease Groups

Percentages of deaths by the cardiovascular disease groups among the total number of deaths by cardiovascular diseases in 2015 in Japan [6]. The cardiovascular disease groups include heart failure, myocardial infarction, the other ischemic heart disease, arrhythmia and the other cardiovascular diseases.

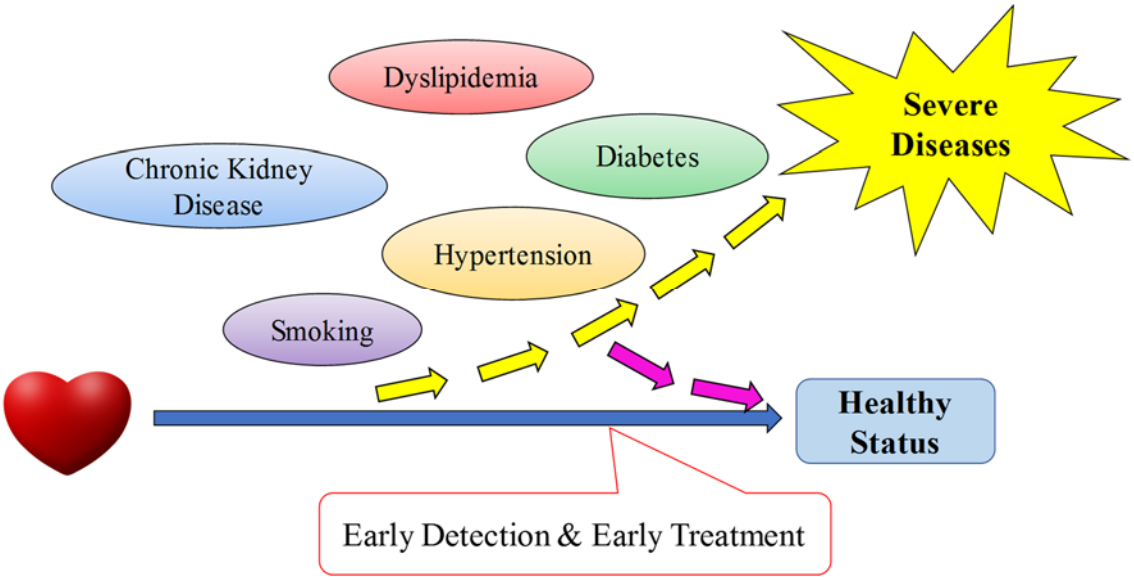

Fig. 2: Significance of Preventive Medicine by Early Detection

Schematic diagram showing the importance of preventive medicine by early detection. Without the early detection and the proper treatment of a sub-clinical cardiovascular disease, it could develop into severe and sometimes fatal diseases such as myocardial infarction or congestive heart failure.

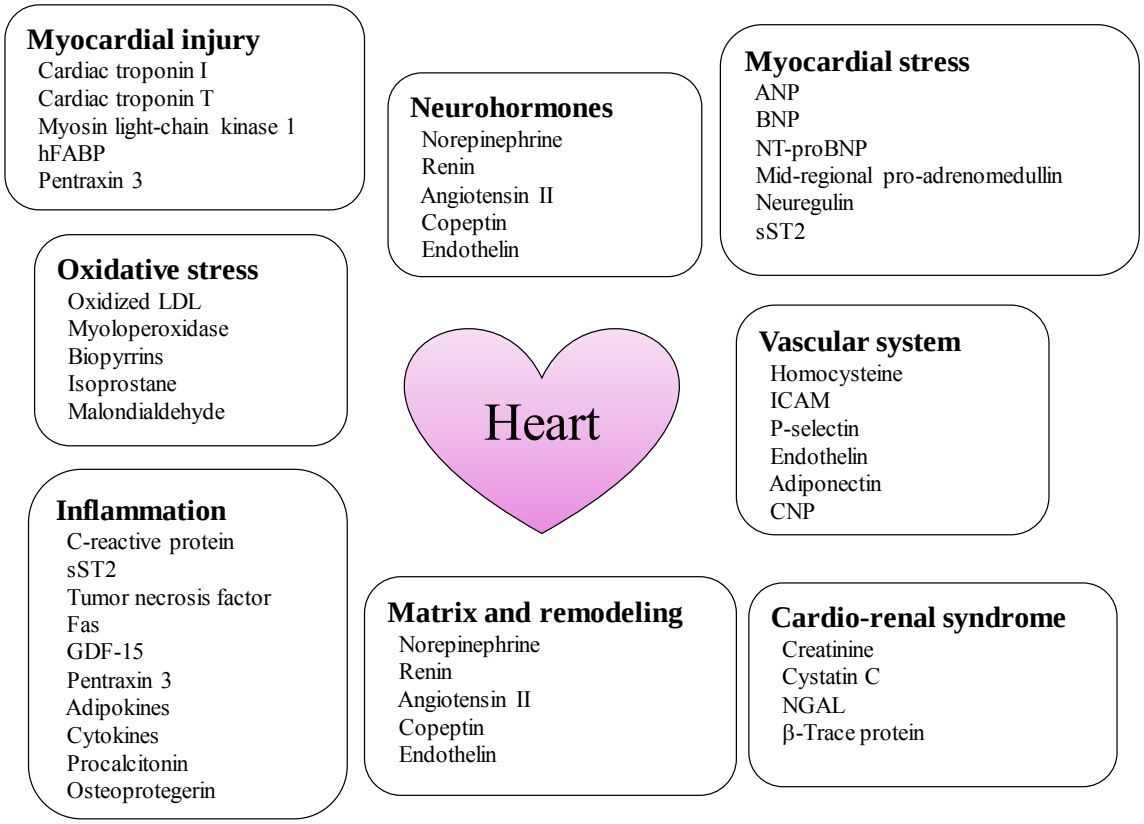

Fig. 3: Biomarkers Related with Cardiovascular Diseases

Schematic diagram of potential biomarkers related with heart diseases group categorized by physiological responses or systems; myocardial injury, oxidative stress, inflammation, neurohormones, matrix and remodeling, myocardial stress, vascular system, and cardio-renal syndrome.

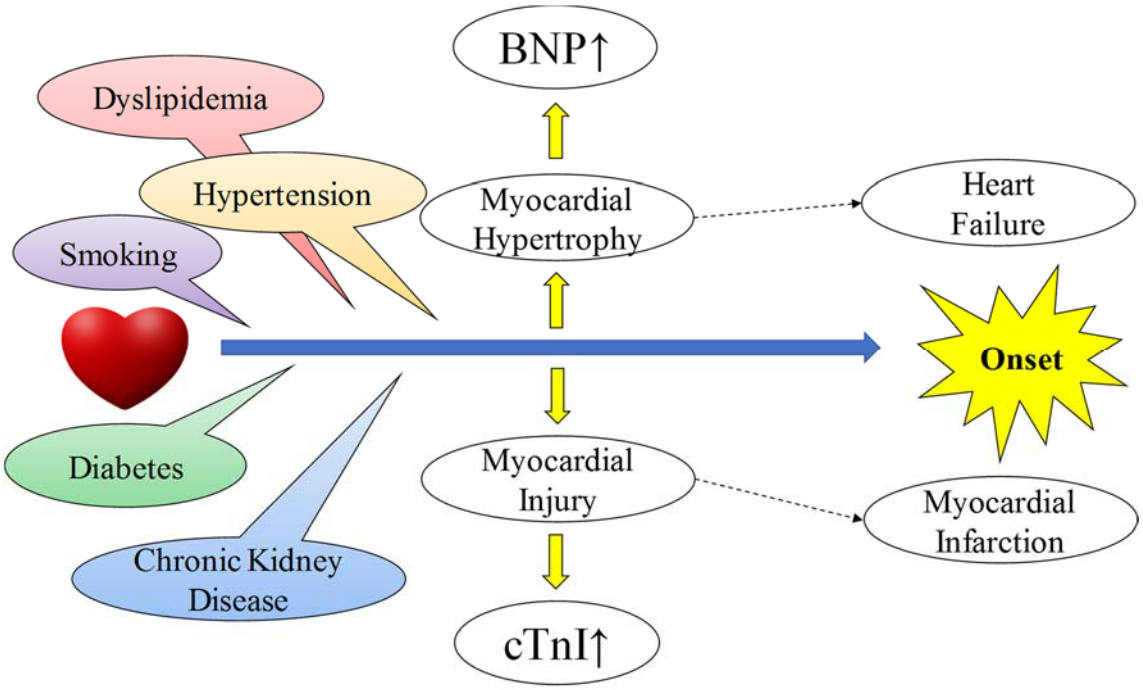

Fig. 4: Detection of Early Signs of Onset by BNP and cTnI

Schematic diagram to show the expected timing of the biomarker elevation. Following the long-term insult by the risk factors such as dyslipidemia, smoking, hypertension, diabetes, or chronic kidney disease, myocardium of the heart could be swollen (hypertrophy) or injured when BNP or cTnI is released into the blood.

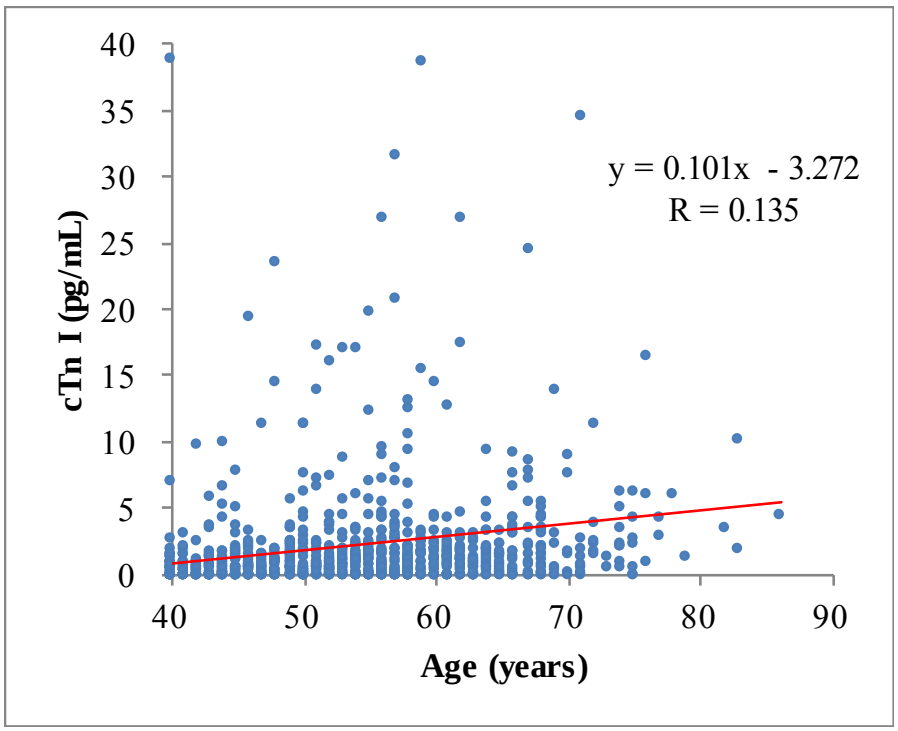

Fig.5: Example of Linear Regression

Example of linear regression illustrated by the correlation between age (X-axis) and cTnI (Y-axis). The regression line obtained by the linear regression is shown in red. The formula of the regression line and the correlation coefficient are shown at upper right.

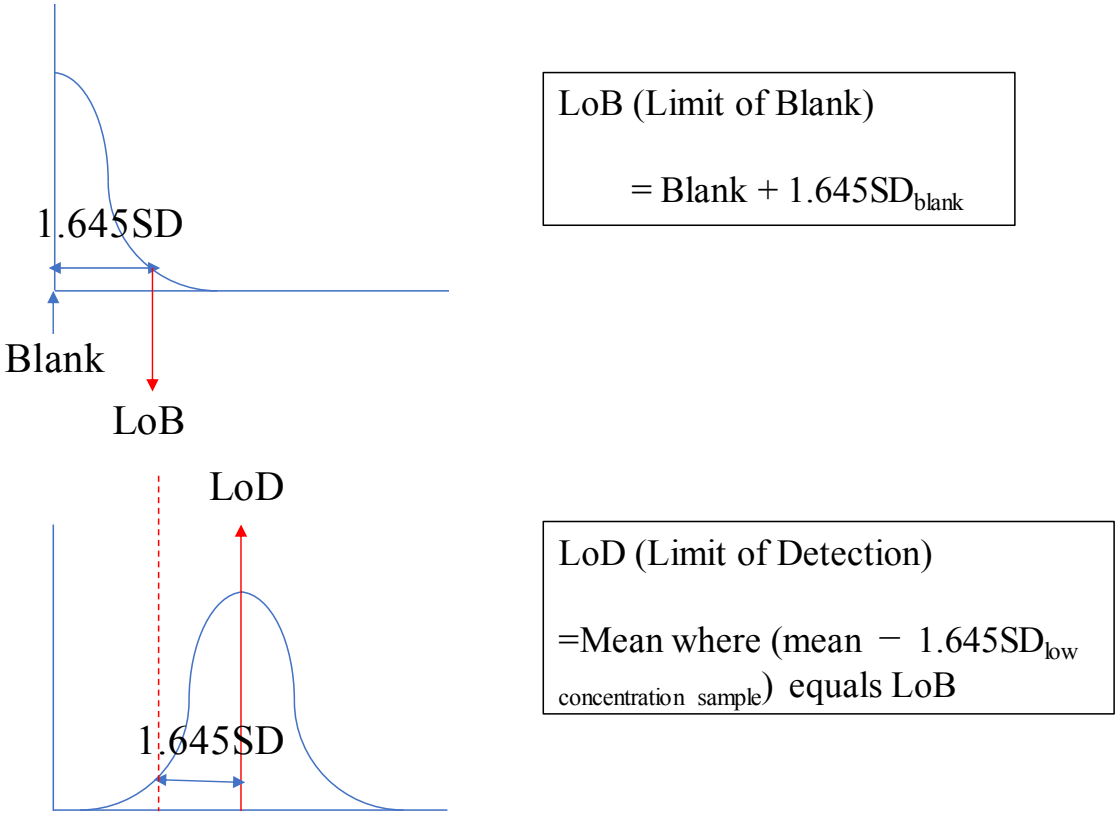

Fig. 6: LoB (Limit of Blank) and LoD (Limit of Detection)

Schematic diagrams to show how LoB (limit of blank) and LoD (limit of detection) are determined. LoB is determined by adding $1.645SD_{blank}$ to blank (upper diagram) while LoD is determined by identifying the mean of low concentration samples where mean – $1.645SD_{low\ concentration\ sample}$ equals the LoB (lower diagram). SD_{blank}: standard deviation of the blank. $SD_{low\ concentration\ sample}$: standard deviation of a given low concentration sample.

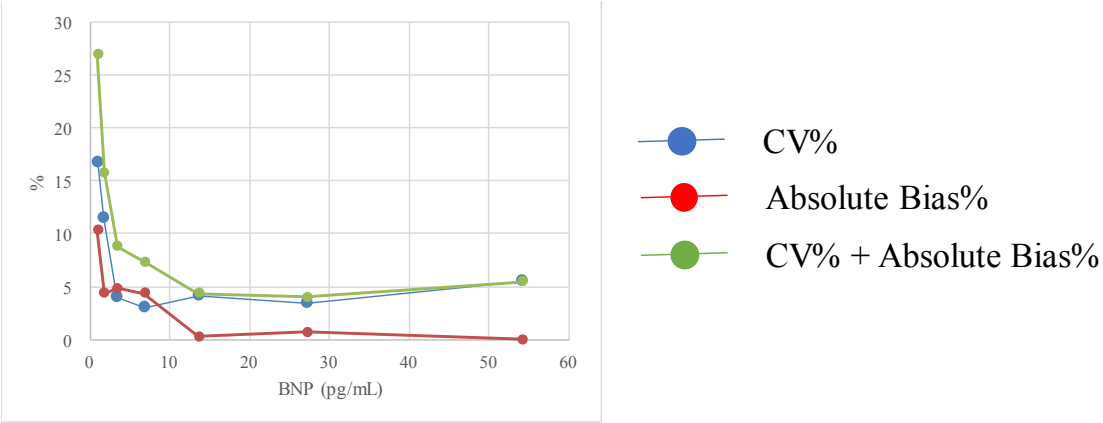

Fig. 7: LoQ (Limit of Quantitation)

Schematic diagram to show how LoQ (limit of quantitation) is determined. For each dilution series sample, sum of CV% and absolute bias% (green) is obtained from CV% (blue) and absolute bias% (red). LoQ is determined by identifying the lowest concentration that shows the sum of CV% and bias% within predefined allowance range (e.g. 30%).

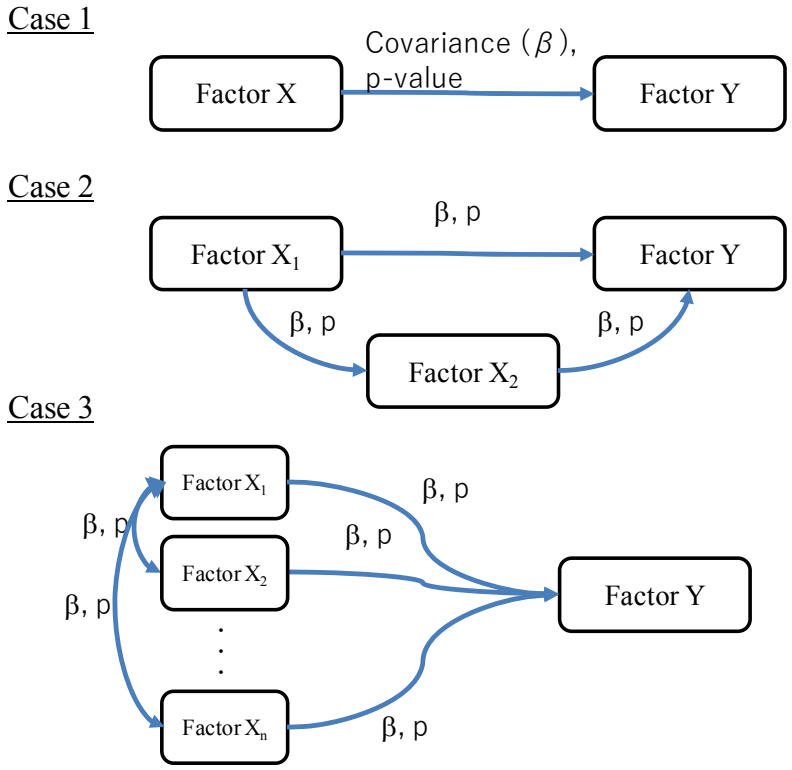

Fig. 8: Covariance Structure Analysis

Schematic diagrams to show how associations between factors (factors X and Y) are analyzed by covariance structure analyses. Case1: the simplest case where association between two factors is analyzed. Case 2: a case where another factor (factor X_2) is added to Case 1. Case 3: a generalized case where multiple factors (factors X_1 through X_n) are analyzed against a factor of interest (factor Y).

Chapter 2. Screening of Cardiovascular Diseases in the General Population with B-type Natriuretic Peptide and cardiac troponin I

1. Introduction

In Chapter 1, I selected BNP and cTnI as the two best biomarkers among the 13 candidate biomarkers for the screening of cardiovascular diseases basing on their desirable specificity and sensitivity in the diagnosis, assessment of the disease severity, monitoring of treatment efficacy and prediction of the future incidences.

BNP, a peptide that was discovered by Sudoh *et al*.[27], is predominantly expressed in ventricles and secreted in response to several factors, including myocardial stretching, increased myocardial pressure, and cell hypoxia, to bring about pharmacological effects, such as vasorelaxation, diuresis, natriuresis, and the inhibition of the renin-angiotensin-aldosterone system [28, 29]. Because the plasma BNP level is correlated with the severity of cardiac dysfunction in patients with heart failure, BNP is used for diagnosis, stratification and monitoring of heart failure in clinical settings [30, 31]. BNP has also been demonstrated to be correlated with the Framingham Risk Score, which is an indicator for the risk of coronary

heart disease in the general population [32].

Troponin I is a protein expressed in myocardium constituting one of three subunits of troponins T, I and C [33]. Because cardiac troponin I (cTnI) is expressed as cardio-specific isoform in the myocardium, it is ideally suited for the detection of myocardial damage [34]. Due to the development of high sensitive troponin assays, the measurement of troponin for the diagnosis of acute coronary syndrome (ACS) is recommended as the most reliable biomarker [35, 36]. High sensitive troponin I assays have been shown to be elevated not only in patients with ACS but also those with chronic cardiovascular diseases including congestive heart failure. This is because high sensitive troponin I assays can detect small amount of cTnI in blood eluted from the myocardium by myocardial injury not only due to ischemic but also due to non-ischemic causes [37]. In fact, it has been shown to be useful not only for the diagnosis of ACS but also for predicting cardiovascular events such as congestive heart failure in patients with stable coronary artery disease [38] or pulmonary hypertension [39]. Zeller *et al*. showed that the high sensitive troponin I assay significantly improved the predictive values of cardiovascular events and coronary deaths in the general population over the contemporary troponin I assay [40].

Due to the different release mechanisms of BNP and cTnI mentioned earlier, I assessed here whether a wider range of clinical and sub-clinical cardiovascular diseases could be detected by screening the general population with a combination of BNP and high sensitive troponin I assays [41]. The objectives of this study therefore will be as follows:

1) To assess whether elevation of BNP or cTnI in the general population is associated with increased cardiovascular risks such as the diseases related to cardiovascular diseases and the Framingham risk factor.

2) To determine whether BNP and cTnI complement with each other in detecting the broader range of high-risk populations.

2. Materials and Methods

1) Study Population

952 subjects who visited Takeda Hospital Medical Examination Center for their annual health evaluation participated in this study. Upon excluding two subjects with the estimated glomerular filtration rate (eGFR) below 30 mL/min/1.73 m^2, 950 subjects were enrolled.

2) Study Design

The study was designed to comply with the Declaration of Helsinki in 1964 and obtained approval of the Institutional Review Board at Takeda Hospital. Informed consent was obtained from each subject before the participation to the study. Adequate care was taken to ensure the protection of the privacy of each subject.

3) Clinical Tests

The clinical tests included body mass index (BMI), heart rate (HR), systolic and diastolic blood pressures (SBP and DBP), cardiothoracic ratio (CTR) determined by echocardiography and vital capacity (VC). Information about gender, age, medical history and smoking habit was collected from all subjects in interviews.

4) Laboratory Tests

All the blood samples were drawn in a sitting position in the morning after the subjects had been fasted since the previous night. All the subjects were evaluated with biochemistry and hematology tests as well as Architect STAT High Sensitive Troponin I (Abbott Laboratories, Illinois, USA) and Architect BNP-JP (Abbott

Laboratories, Illinois, USA) tests. The biochemistry test was performed on JCA-8060 (JEOL Ltd., Tokyo, Japan) that included albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), uric acid (UA), creatinine (CRE), blood urea nitrogen (BUN), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), hemoglobin A1c (HbA1c) and fasting blood glucose (FBG). The hematology test was performed on E-2100 (Sysmex Corporation, Kobe, Japan) that included white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht) and platelet count (PLT). The eGFR was calculated according to the revised equations for the Japanese population as reported previously [42].

5) Calculation of the FRS

The FRS was calculated according to the methods of D'Agostino *et al* [13].

6) Association with Diseases Related with Cardiovascular Risks

I defined diseases related with cardiovascular risks that included hypertension, dyslipidemia, diabetes, chronic kidney disease (CKD) and hyper-uricemia

according to the following criteria. The presence of hypertension was defined when a subject was treated for the disease, the SBP was more than 139 mmHg or the DBP was more than 89. The presence of dyslipidemia was defined when a subject was treated for the disease or had steatosis, a high-density lipoprotein cholesterol (HDL-C) level less than 40 mg/dL, an LDL-C level more than 139 mg/dL or a TG level more than 149 mg/dL. The presence of diabetes was defined when a subject was treated for the disease, had a fasting blood glucose (FBG) level more than 125 mg/dL or an HbA1c value more than 6.4%. The presence of CKD was defined when a subject was treated for the disease, had an eGFR less than 60 mL/min/1.73 m^2 or had positive urine protein results. The presence of hyper-uricemia was defined when a subject was treated for the disease, had gout, or their uric acid (UA) level was more than 6.9 mg/dL.

7) Comparisons of BNP-cTnI Quadrants

Using the clinical cut-off values of BNP [43] and cTnI [44], I defined quadrants according to BNP and cTnI levels as follows: (i) Quadrant A: BNP level equal to or less than 40.0 pg/mL and cTnI level more than 26.2 pg/mL, (ii) Quadrant B: BNP level equal to or less than 40.0 pg/mL and cTnI level equal to or less than

26.2 pg/mL, (iii) Quadrant C: BNP level more than 40.0 pg/mL and cTnI level equal to or less than 26.2 pg/mL, (iv) Quadrant D: BNP level more than 40.0 pg/mL and cTnI level more than 26.2 pg/mL. With these quadrants, I assessed factors that differentiated pairs of quadrants by performing Wilcoxon's test. For the assessment, I chose age, BMI, SBP, HR, CTR, VC, Hb, PLT, UA, eGFR, BUN, LDL-C, HDL-C, TG, HbA1c, FBG as the candidate variables for cardiovascular risks. I also included the FRS to confirm whether quadrants A and C were associated with the FRS.

8) Statistical Analyses

JMP 11.0.0 (SAS) was used for the statistical analyses. The 95th percentiles of the distribution of BNP levels and the 99th percentiles of the distribution of cTnI levels in the population in this study were determined using the robust statistical method described in the Clinical & Laboratory Standards Institute (CLSI) document C28-A3c [45]. The differences of the basic characteristics by gender were assessed by Wilcoxon's test. The associations of BNP or cTnI with the FRS were assessed by univariable followed by multivariable linear regression analyses in each gender. The associations of BNP or cTnI level with the diseases related

with cardiovascular risks were assessed by multivariable linear regression analyses. For these analyses, the presence or absence of a disease was encoded as "0 (absent)" or "1 (present)."

3. Results

1) Characteristics of the Subjects

I summarized the background characteristics of the 950 subjects in Table 1. All the parameters were significantly different by gender except for the age, CTR, eGFR, LDL-C, HbA1c, and the presence of CKD.

2) Association with the FRS

The associations of the BNP or cTnI level with the FRS in each gender by the linear regression analyses were shown in Table 2. The BNP and cTnI levels were significantly and independently associated with the FRS in both genders.

3) Association with Diseases Related with Cardiovascular Risks

By the multivariable linear regression analyses, the presence of hypertension and

CKD were positively, but that of dyslipidemia was negatively associated with the BNP level, while the presence of hypertension and dyslipidemia were positively associated with the cTnI level (Table 3).

4) Comparisons of BNP-cTnI Quadrants

By the definition described in the Methods, I obtained 9, 932, 9 and 0 subjects in quadrant A, B, C and D, respectively (Fig. 1). I then assessed the differences between pairs of quadrants among quadrants A, B and C using Wilcoxon's test (Table 4, Fig. 2). The BMI, CTR and the FRS were significantly higher in quadrant A than quadrant B. The age, CTR and the FRS were significantly higher and the PLT were significantly lower in quadrant C than quadrant B. Between quadrant A and C, the age was significantly lower, but the BMI and TG were significantly higher in quadrant A than those in quadrant C.

4. Discussions

In this study, I assessed the validity of the combination assay of BNP and cTnI for the screening of cardiovascular risks in the general population. In the background characteristics by gender, there were significant differences between the

gender (Table 1). As for most of the parameters related with cardiovascular risks including BMI, blood pressure, lipids and HbA1c, higher cardiovascular risks were indicated in the male group in comparison with the female group. The prevalence of the diseases related with cardiovascular diseases and the FRS were also higher in the male group. In consistent with these results, the cTnI level was higher in the male group as shown in previous reports [46, 47, 48]. The BNP level, on the contrary, was lower in the male group, which is consistent with the report by Kawai *et al*. [43]. Considering the result by Kawai *et al.* that the BNP level was negatively correlated with BMI [43], I thought the result in Table 1 that BMI was higher in the male group and the result in Table 3 that the BNP level was negatively correlated with dyslipidemia, were consistent.

The result in Table 2 showed that the cTnI and BNP levels were positively and independently associated with the FRS. From this result, I think both cTnI and BNP are the indicators of risk for cardiovascular risk but they reflect different aspects of cardiovascular disorders.

In Table 3, BNP was significantly correlated with hypertension, dyslipidemia and CKD, but the correlation with dyslipidemia was inversed. On the other hand, both hypertension and dyslipidemia were positively correlated with cTnI. I

understand this result as that hypertension is the common factor to elevate cTnI and BNP while dyslipidemia being the differentiating factor to decide whether cTnI or BNP is elevated.

In Table 4 and Fig. 2, I characterized quadrants A, B and C. The result that quadrants A and C showed significantly higher CTR and FRS than quadrant B was thought to be one of the evidences to show the association of quadrants A and C with high cardiovascular risks. I interpreted the reason why BNP and cTnI were independently associated with the FRS as shown in Table 2 was because cTnI and BNP detect the two different populations (quadrant A and C). The factors that differentiated quadrants A and C were age and BMI. Together with the results in Table 3 that the BNP level was inversely associated with dyslipidemia while the TnI level was positively associated with, it could be assumed that obesity is a key factor that differentiates quadrants A and C. Because of the suppressed BNP level in quadrant A, the heart may be less protected due to attenuated cardio-protection by BNP and more susceptible to myocardial injury, giving ground to the TnI elevation. The result that the median of the age in quadrant A (59.0) was significantly lower than that in quadrant C (71.0) may indicate cardiac diseases related with dyslipidemia (e.g. atherosclerosis, angina pectoris, or myocardial infarction) develop

earlier in life than those without one.

Taking all these results into consideration, I constructed a putative pathway of how these risk factors contribute in the elevation of BNP or cTnI level (Fig. 3). The idea is that hypertension is the common factor for the elevation of BNP and cTnI while dyslipidemia is a factor to differentiate the pathway of BNP or cTnI elevation, and CKD is a trigger for BNP elevation. From subject's perspective, subjects with hypertension and dyslipidemia could develop myocardial injury as represented by the elevation of cTnI in their comparatively early age (e.g. in their 50's) while subjects with hypertension but without dyslipidemia would evade developing myocardial injury and live longer but may develop cardiac hypertrophy as represented by the elevation of BNP in their elder age (e.g. in their 70's).

Table 1. Background Characteristics of the Subjects.

	Unit	Female (N = 439)	Male (N = 511)	P-value
		Median (25%ile, 75%ile)	Median (25%ile, 75%ile)	
Age	years	53.0 (46.5, 60.0)	54.0 (48.0, 61.0)	0.079
BMI	kg/m^2	21.4 (19.5, 23.5)	23.3 (21.6, 25.5)	< 0.001
SBP	mmHg	114.0 (103.0, 125.5)	120.0 (111.0, 132.0)	< 0.001
DBP	mmHg	72.0 (64.0, 81.0)	79.0 (71.0, 88.0)	< 0.001
HR	bpm	73 (67, 80)	70 (63, 78)	< 0.001
CTR	%	44.4 (41.3, 47.6)	44.7 (41.7, 47.5)	0.335
VC	L	2.9 (2.5, 3.2)	4.1 (3.7, 4.5)	< 0.001
WBC	/uL	4800 (4000, 5600)	5300 (4450, 6350)	< 0.001
RBC	10^4/uL	437 (417, 459)	483 (459, 510)	< 0.001
Hb	g/dL	13.1 (12.4, 13.6)	14.9 (14.2, 15.5)	< 0.001
Ht	%	40.1 (38.1, 41.6)	45.0 (43.0, 46.8)	< 0.001
PLT	10^4/uL	23.6 (20.7, 26.6)	22.5 (19.4, 25.8)	< 0.001
ALB	g/dL	4.4 (4.3, 4.6)	4.5 (4.3, 4.7)	< 0.001
AST	U/L	20.0 (18.0, 24.0)	22.0 (18.5, 26.0)	< 0.001
ALT	U/L	17.0 (14.0, 22.0)	22.0 (17.0, 30.0)	< 0.001
GGT	U/L	18.0 (14.0, 24.5)	32.0 (22.0, 55.5)	< 0.001
UA	mg/dL	4.5 (3.9, 5.0)	6.1 (5.2, 6.8)	< 0.001
eGFR	mL/min/1.73 m^2	70.8 (63.5, 79.6)	71.5 (65.1, 78.0)	0.792
BUN	mg/dL	13.0 (11.0, 16.0)	14.0 (12.0, 16.0)	< 0.001
LDL-C	mg/dL	122.0 (102.5, 142.0)	125.0 (106.0, 146.0)	0.220
HDL-C	mg/dL	76.0 (64.0, 87.0)	59.0 (50.0, 70.0)	< 0.001
TG	mg/dL	71.0 (55.0, 99.0)	102.0 (73.0, 146.5)	< 0.001
HbA1c	% (NGSP)	5.6 (5.5, 5.8)	5.7 (5.5, 5.9)	0.119
FBG	mg/dL	95.0 (90.0, 100.0)	101.0 (95.0, 108.0)	< 0.001
FRS		6.0 (3.0, 10.0)	10.0 (7.0, 13.0)	< 0.001
CKD	%	16.4	14.9	0.518
Dyslipidemia	%	39.9	64.0	< 0.001
Hypertension	%	22.8	38.6	< 0.001
Diabetes	%	3.9	8.0	0.008
Hyper-uricemia	%	0.9	22.1	< 0.001
Current smoker	%	5.9	24.3	< 0.001
		95%ile (95% CI)	95%ile (95% CI)	
BNP	pg/mL	23.1 (21.0, 28.9)	17.8 (15.6, 23.7)	< 0.001
		99%ile (95% CI)	99%ile (95% CI)	
cTnI	pg/mL	23.5 (11.5, 136.0)	26.8 (17.5, 65.2)	< 0.001

Table 2. Univariable and Multivariable Linear Regression Analyses for the Association of BNP or TnI with the FRS.

		Univariable				Multivariable		
		Beta	SE	P-value		Beta	SE	P-value
Females					Intercept	5.969	0.284	< 0.001
	BNP	0.095	0.024	< 0.001	BNP	0.088	0.023	< 0.001
	cTnI	0.092	0.025	< 0.001	cTnI	0.083	0.025	< 0.001
Males					Intercept	9.197	0.267	< 0.001
	BNP	0.148	0.026	< 0.001	BNP	0.140	0.025	< 0.001
	cTnI	0.157	0.038	< 0.001	cTnI	0.142	0.037	< 0.001

Table 3. Multivariable Linear Regression Analyses for the association of BNP or cTnI with Diseases Related with Cardiovascular Risks.

	BNP			cTnI		
	Beta	SE	P-value	Beta	SE	P-value
Intercept	8.054	0.406	< 0.001	0.908	0.336	0.007
Hypertension	1.938	0.571	0.001	1.971	0.472	< 0.001
Dyslipidemia	-2.347	0.533	< 0.001	1.081	0.441	0.014
Diabetes	0.845	0.880	0.338	-0.761	0.729	0.296
CKD	1.869	0.711	0.009	0.359	0.588	0.541
Hyper-uricemia	-1.259	0.796	0.114	0.798	0.659	0.226

Table 4. Assessment of Differentiating Factors Between Quadrants A, B and C.

P-value less than 0.05 was shown in red.

	Quadrant A N = 9	Quadrant B N = 932	Quadrant C N = 9	P-value		
	Median (25%ile, 75%ile)	Median (25%ile, 75%ile)	Median (25%ile, 75%ile)	A vs. B	B vs. C	C vs. A
Gender	-	-	-	0.915	0.915	1.000
Age	59.0 (56.0, 68.0)	54.0 (47.0, 60.0)	71.0 (68.0, 77.0)	0.120	< 0.001	0.047
BMI	25.6 (23.8, 25.8)	22.5 (20.6, 24.6)	20.1 (19.8, 24.5)	0.010	0.218	0.027
SBP	122.0 (115.0, 128.0)	117.0 (107.0, 128.0)	119.0 (113.0, 130.0)	0.466	0.407	0.965
HR	78.0 (63.0, 87.0)	71.0 (65.0, 79.0)	77.0 (72.0, 84.0)	0.560	0.242	0.860
CTR	46.3 (45.9, 49.1)	44.6 (41.5, 47.6)	47.2 (44.2, 53.6)	0.028	0.026	0.894
VC	3.5 (2.9, 4.0)	3.4 (2.9, 4.1)	3.7 (2.1, 3.9)	0.787	0.463	0.659
Hb	13.9 (13.9, 15.1)	14.0 (13.1, 15.0)	13.9 (12.8, 14.3)	0.759	0.636	0.689
PLT	22.2 (19.4, 23.6)	23.0 (20.2, 26.1)	18.9 (15.7, 20.0)	0.241	0.002	0.145
UA	6.1 (4.8, 6.7)	5.2 (4.4, 6.3)	5.7 (4.5, 6.3)	0.324	0.898	0.452
eGFR	73.9 (65.9, 81.3)	71.2 (64.6, 78.6)	65.7 (55.8, 77.2)	0.593	0.217	0.216
BUN	14.0 (13.0, 15.0)	14.0 (11.0, 16.0)	14.0 (13.0, 17.0)	0.585	0.368	0.789
LDL-C	124.0 (114.0, 135.0)	124.0 (104.0, 145.0)	114.0 (96.0, 126.0)	0.939	0.196	0.249
HDL-C	57.0 (54.0, 65.0)	67.0 (55.0, 80.0)	63.0 (56.0, 81.0)	0.081	0.876	0.250
TG	127.0 (75.0, 237.0)	86.0 (61.8, 124.3)	84.0 (61.0, 97.0)	0.057	0.390	0.038
HbA1c	5.8 (5.7, 6.0)	5.6 (5.5, 5.9)	5.7 (5.4, 5.7)	0.158	0.971	0.418
FBG	98.0 (96.0, 106.0)	97.0 (92.0, 105.0)	104.0 (95.0, 108.0)	0.446	0.222	0.724
FRS	12.0 (11.0, 14.0)	8.0 (6.0, 12.0)	13.0 (12.0, 17.0)	0.006	0.002	0.561

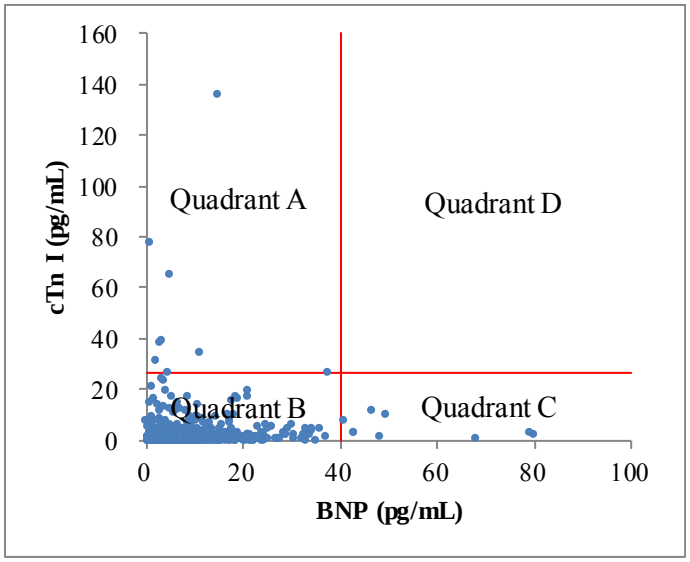

Fig. 1: Graphical Representation of Two-dimensional Distribution of BNP and cTnI.

Quadrants A, B, C and D are separated by cut-off of BNP (40 pg/mL) and that of cTnI (26.2 pg/mL). Quadrant A: upper left, quadrant B: lower left, quadrant C: lower right, and quadrant D: upper right.

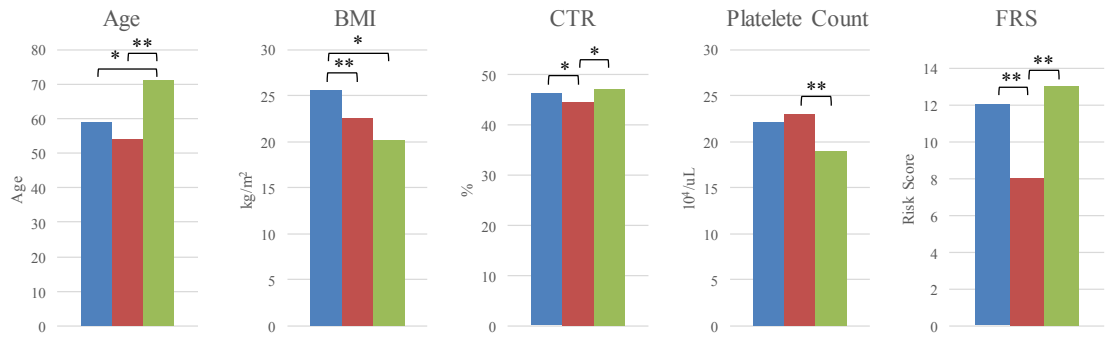

Fig. 2: Graphical Representation of Wilcoxon's Test Shown in Table 4.

Factors (age, BMI, CTR, platelet count and FRS) in Quadrants A, B and C were assessed by Wilcoxon's test. Blue bar: quadrant A. Red bar: quadrant B. Green bar: quadrant C. *: p-value < 0.05. **: p-value < 0.01.

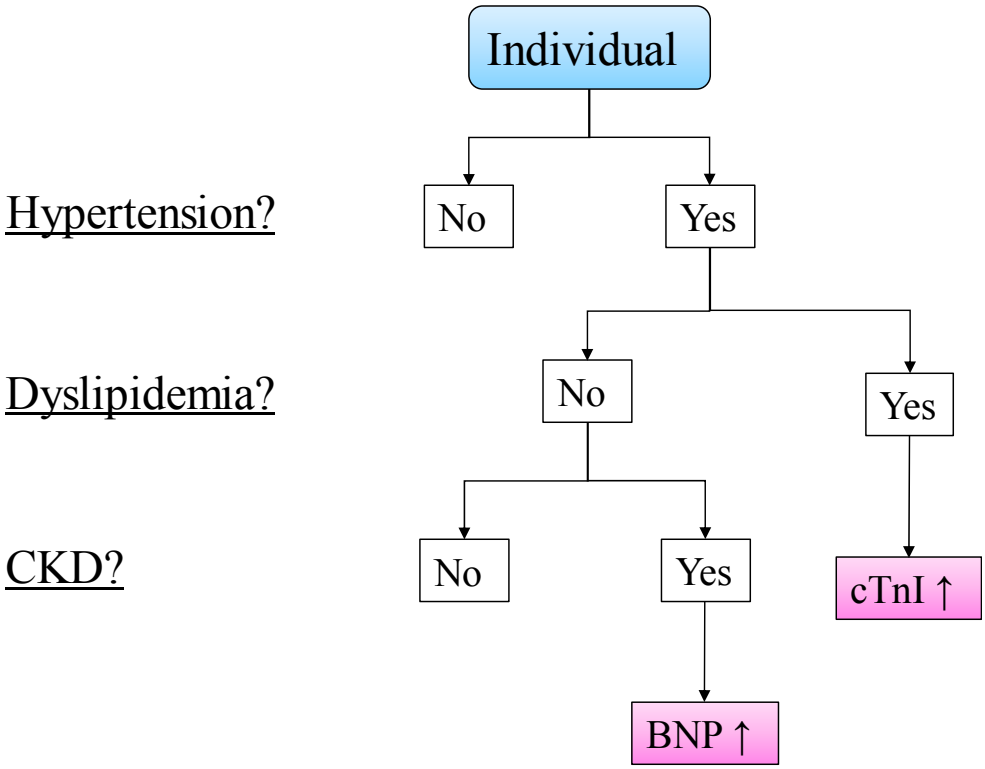

Fig. 3: Speculated Pathway of the Biomarker-Elevation

Schematic diagram to show the pathway of biomarker-elevation speculated from the results in Chapter 2.

Chapter 3. Increased Levels of Cardiac Troponin I in Subjects with Extremely Low B-type Natriuretic Peptide Levels

1. Introduction

In Chapter 2, I have confirmed that BNP and cTnI detect populations with high cardiovascular risks in the general population and that BNP and cTnI detect non-overlapping two different high-risk populations at both wings of the characteristic “L-shaped distribution” in the 2D plot (Chapter 2, Fig. 1). In this Chapter, I examined whether BNP and cTnI influence each other in forming this distribution, especially as to whether elevation of cTnI level is associated with suppressed BNP level.

The BNP cut-off level was reported at 18.4 pg/mL in 1993 [49], but 40 pg/mL was proposed in 2012 as an alternative cut-off for practical use in clinical settings [43]. In either case, there has been a basic understanding that lower BNP levels indicate better prognoses (Fig. 1). Among the numerous previous reports on BNP, I could not find any article that focused on the biological implications of extremely low BNP levels (e.g., BNP levels below 4 pg/mL), which I examined in this study.

As mentioned in Chapter 2, cTnI is expressed in the myocardium as a cardio-specific isoform, and thus it is ideally suited as a marker of myocardial damage [34]. Owing to the development of high sensitive troponin assays in recent years, the measurement of troponin levels with those assays for the diagnosis of acute coronary syndrome (ACS) is stipulated as more reliable than measurements with any other conventional biomarkers based on latest guidelines [35, 36]. High sensitive cTnI assays such as the one used in this study are not only used for the diagnosis of ACS but also known to have the ability to predict cardiovascular events in the general population. By measuring cTnI levels with the high sensitive cTnI assay in a Scottish cohort consisting of 15,340 individuals from the general population, Zeller *et al*. showed that cTnI was associated with future cardiovascular events over an average of 20 years of follow-up and suggested threshold values of 4.7 pg/mL for women and 7.0 pg/mL for men to identify individuals at risk of future cardiovascular events [40]; these results suggested threshold values are far below the 99th percentiles of cTnI concentrations shown in the package insert of the assay – 15.6 pg/mL for women and 34.2 pg/mL for men.

In a 10-year follow-up of the Inter99 study, Hansen *et al*. showed with a cohort of 6,238 general population that metabolically healthy obese subjects had a higher

risk of ischemic heart disease (IHD) than metabolically healthy subjects with normal weight [50]. As shown by several reports, BNP level is reduced in individuals with obesity independent of the presence or absence of cardiac diseases [51-53]. The metabolically healthy obese subjects in the Inter99 Study are therefore considered to be equivalent to individuals with reduced BNP levels. Because of the cardio-protective effects exerted by BNP, Tsutsumi *et al.* hypothesized that the increased risk of IHD in individuals with obesity is due not only to well-known risk factors (e.g., hypertension, dyslipidemia, diabetes) but also to the compromised cardio-protection of the reduced BNP level as shown in Fig. 2 [54]. By a covariance structure analysis in 1,252 patients with cardiac disorders, they confirmed that low BNP level, as well as hypertension, dyslipidemia and haemoglobin A1c (HbA1c), but not body mass index (BMI) was significantly associated with the incidence of IHD ($p < 0.001$) [54]. To confirm this hypothesis further, I assessed whether low BNP level was associated with an enhanced cardiovascular risk in the general population in this study by using cTnI as an indicator of cardiac disorders [55].

2. Materials and Methods

1) Study Population

I conducted this study in collaboration with Takeda Hospital Medical Examination Center and Niigata Medical Association of Occupational Health. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Takeda Hospital Institutional Review Board and the Ethics Committee at Niigata Medical Association of Occupational Health. Upon obtaining informed consent, I recruited 2,005 subjects older than 38 years who visited Takeda Hospital Medical Examination Center or Niigata Medical Association of Occupational Health for their annual health check-up and showed no apparent symptoms of cardiac disease during doctor interviews. Among the recruited subjects, I excluded subjects with outlying laboratory test results by Dixon's method [23] and subjects with an eGFR below 30 mL/min/1.73 m^2. This resulted in the exclusion of one subject who exhibited an extremely high cTnI level (826.1 pg/mL) and three subjects who exhibited eGFR below 30 mL/min/1.73 m^2, corresponding to the enrolment of 2,001 subjects (884 females and 1,117 males) in total.

2) Clinical and Laboratory Tests

All subjects underwent routine biochemical and hematological analyses for their health check-ups. BNP level was measured by an ARCHITECT BNP-JP assay (Abbott Laboratories, Abbott Park, IL, USA). According to the package insert of the BNP assay, the analytical sensitivity, which is defined as the concentration of the mean of the blank + 2SD, was 5.8 pg/mL. cTnI level was measured with an ARCHITECT STAT High Sensitive troponin I assay (Abbott Laboratories, Abbott Park, IL, USA) [44]. According to the package insert of the cTnI assay, the 99th percentile of cTnI level in an apparently healthy population was 26.2 pg/mL, the LoD was 1.9 pg/mL, and the CV% at the 99th percentile was 4.0%. All blood samples were drawn from participants in a sitting position on the following morning after they had fasted overnight. BMIs were calculated based on the subjects' heights and weights. The information regarding gender, age, medical history and smoking habits was collected from interviews. The estimated glomerular filtration rate (eGFR) was calculated using equations for the Japanese population, as reported previously [42]. The Framingham Risk Score was calculated according to the D'Agostino's formula [13].

Chronic kidney disease (CKD) was defined as present when a subject was under treatment for the disease, had an eGFR less than 60 mL/min/1.73 m^2 or

had positive urine protein results. Dyslipidaemia was defined as present when a subject was under treatment for the condition, had steatosis, had a high-density lipoprotein cholesterol (HDL-C) level lower than 40 mg/dL, had a low-density lipoprotein cholesterol (LDL-C) level higher than 139 mg/dL or had a triglyceride (TG) level higher than 149 mg/dL. Hypertension was defined as present when a subject was under treatment for the condition, had a systolic blood pressure (SBP) higher than 139 mmHg or had a diastolic blood pressure (DBP) higher than 89 mmHg. Diabetes was defined as present when a subject was under treatment for the disease, had a fasting blood glucose (FBG) level higher than 125 mg/dL or had an HbA1c value (NGSP) higher than 6.4%.

3) Validation of the BNP Assay

To assess the lowest quantifiable BNP level of the BNP assay used here, I performed a reproducibility test with serially diluted samples. The samples were prepared by serially diluting Control L (BNP = 54.2 pg/mL) with Calibrator A (BNP = 0.0 pg/mL), which are components of the BNP assay, by twofold down to BNP level below 1 pg/mL. The reproducibility test was performed by repeating the measurements ten times for each sample. I defined the LoQ as the

lowest BNP concentration for which the total error, defined as the sum of the CV% and absolute bias, was equal to or less than 30% based on reports in the literature [25]. The absolute bias was defined as the absolute value of the bias, which was the percentage of deviation of the measured value from the expected value.

4) Metabolic Derangement in the Low BNP Subgroups

To evaluate the presence of metabolic derangement in patients with extremely low BNP levels, I investigated the percentage of subjects who exceeded the ULN WC or BMI in the low BNP subgroups. According to the report from the Japan Society for the Study of Obesity, 90 cm for women and 85 cm for men were used as the ULN for WC and 25 kg/m^2 was used as the ULN for BMI [56]. The low BNP subgroups were obtained from the total cohort by limiting the maximum BNP level to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 pg/mL.

5) Statistical Analyses

I used JMP 11.0.0 (SAS Institute Inc., Cary, NC, USA) for multivariate analyses. To analyze the factors that contributed to the elevation of cTnI level, I

used IBM SPSS Amos (IBM, Armonk, New York, USA) to perform covariance structure analysis by fitting the data into structural equation models (SEM), with which one of my collaborators, Professor Yoshimura at the Jikei University, had successfully elucidated the relationship among factors involved in cardiovascular pathogenesis [54, 57-61]. For the analyses, I converted BNP and cTnI into log(BNP) and log(cTnI) respectively because the distributions were non-Gaussian when confirmed by Shapiro-Wilk tests. For the purpose of the analyses, I assigned cTnI values lower than 0.1 pg/mL to 0.05 pg/mL. For the assessment of the parameters that contributed to cTnI elevation, I chose log(BNP), gender, age, BMI, SBP, eGFR and Hb as the possible risk factors and categorized the rest of the factors into e1.

To assess the mechanism by which cTnI levels were associated with BNP levels in the low BNP subgroups, I defined the subgroups among the overall population by limiting the maximum BNP level to cut-off values of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 20 pg/mL and performed covariance structure analyses for each subgroup. P values of < 0.05 were considered as statistically significant.

3. Results

1) Characteristics of the Study Population

The characteristics of the total cohort (884 females and 1,117 males) are presented in Table 1. Briefly, the median values of the age, BMI, BNP level and cTnI level of the subjects were 56.0 years, 22.6 kg/m^2, 5.6 pg/mL and 1.5 pg/mL, respectively. The median Framingham Risk Score was 10.0, which meant a 6.3% 10-year cardiovascular risk for the women and a 9.4% risk for the men according to a previous report [13].

2) Analytical Performance of the BNP Assay

I performed a reproducibility test for the BNP assay used in this study and assessed the limit of quantitation (LoQ) as described in Materials and Methods. As shown in Table 2, the total error, defined as the sum of the coefficient of variation (CV%) and absolute bias, was within 30% when assessed with serially diluted samples of BNP levels ranging from 0.8 pg/mL to 54.2 pg/mL. From this result, the LoQ of the BNP assay was determined to be 0.8 pg/mL.

3) Two-dimensional Distribution of BNP and cTnI

A two-dimensional distribution chart was constructed by plotting BNP level on the X-axis and cTnI level on the Y-axis for the total cohort (N = 2,001) as illustrated in Fig. 3. The correlation coefficient of the linear regression of the distribution was 0.096 ($p < 0.001$), and the regression formula was $Y = 0.090X + 0.239$.

4) Metabolic Derangement in the Low BNP Subgroups

To evaluate the presence of metabolic derangement in patients with extremely low BNP levels, I determined the percentage of subjects who exceeded the upper limit of normal (ULN) WC or BMI. As shown in Fig. 4, drastic increases in the percentage of subjects exceeding the ULN WC and BMI were seen in subgroups with BNP levels lower than 4 pg/mL.

5) Covariance Structure Analyses

I performed covariance structure analyses with the 12 low BNP subgroups with the upper limits of BNP levels at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 pg/mL. The numbers of subjects in these subgroups were 81, 287, 516, 730, 918, 1,070, 1,205, 1,329, 1,416, 1,490, 1,747 and 1,860, respectively. The covariance

structure analyses were performed to assess factors associated with elevated log(cTnI) within these subgroups. The beta value between log(BNP) and log(cTnI) levels was negative (-0.29) in the subgroup with the BNP cut-off levels at 1 pg/mL (Fig. 5a, N = 81) but positive (0.04) in the subgroup with the BNP cut-off level at 20 pg/mL (Fig. 5b, N = 1,860). Fig. 6 shows the plots of beta values of all the subgroups in the Y-axis against BNP cut-off levels in the X-axis. A sharp drop in the beta value was observed as the BNP cut-off level decreased from 20 pg/mL to 1 pg/mL. In particular, the beta values for the subgroups with BNP cut-off levels at 1, 2, and 3 pg/mL demonstrated strong significance ($p < 0.005$).

6) Multivariate Analyses

To evaluate the contribution of obesity to the elevation of cTnI level, I performed multivariate analyses for log(cTnI) with the total cohort and with the subgroup with BNP level lower than 4 pg/mL (Table 3). In similar to the results of the covariance structure analyses, the coefficient of log(BNP) against log(cTnI) in the subgroup with BNP level lower than 4 pg/mL was inversed (-0.423), whereas it was positive (0.149) in the total cohort. In the subgroup with

BNP level lower than 4 pg/mL, BMI was not significantly associated with log(cTnI).

4. Discussions

In this study, I confirmed that BNP was positively correlated with cTnI in the total cohort (Fig. 3) and that this positive correlation was maintained in the group with the BNP cut-off level at 20 pg/mL (Fig. 5b). I think that this is consistent with previous reports that showed the elevation of cTnI in subjects with chronic cardiac diseases, including congestive heart failure, left ventricle hypertrophy, and cardiomyopathy [37], in which case BNP level is usually elevated, as well as the elevation of BNP in subjects with acute myocardial infarction [62], in which case cTnI level is usually elevated.

Regarding the BNP assay that I employed in this study, 5.8 pg/mL was the lowest measurable BNP concentration according to the package insert. For the purpose of this study, however, I confirmed that the LoQ was 0.8 pg/mL by the definition described in Materials and Methods (Table 2). I then proceeded to define the subgroups with BNP cut-off levels as low as 1 pg/mL, which provided a basis for the breakthrough in elucidating the pathogenic role of low BNP level. By

performing covariance structure analyses using subgroups with BNP cut-offs equal to or lower than 20 pg/mL, I confirmed that the beta values of log(BNP) against log(cTnI) decreased as the BNP cut-off levels decreased from 20 pg/mL to 1 pg/mL, and the inverse correlation became significant ($p < 0.005$) at BNP cut-off levels lower than 4 pg/mL (Fig. 6). From these results, I concluded that subjects with BNP levels lower than 4 pg/mL were susceptible to myocardial injury due to decreased BNP level. These results are consistent with the findings of the previous report by Tsutsumi *et al.* that low BNP level was significantly associated with the incidence of IHD ($p < 0.001$) [54] and support the hypothesis that insufficient BNP level may play a pathogenic role in the occurrence of cardiac disorders.

Notably, the association between BMI and log(cTnI) in the low-BNP group was not significant (Table 3 and 4). Because increased BMI is reported to be associated with reduced BNP level [51-53], the inverse association between cTnI and BNP may indicate that cTnI elevation did not directly result from reduced BNP level but rather from increased BMI, which coincidentally reduced BNP level. However, the results demonstrated that the elevation in log(cTnI) was inversely and directly associated with BNP but not with BMI in the low-BNP group (Table 3 and 4), whereas the elevation in log(cTnI) was positively associated with both BNP and

BMI in the control group (Table 3 and 5). This result is consistent with the previous report that obesity contributes to the incidence of IHD via low BNP levels [54].

Pleiotropic cardio-protective effects exerted by BNP have been reported by several researchers [63]. These effects include natriuresis, diuresis, vasodilation, lusitropy, lipolysis, weight loss and improved insulin resistance. As mentioned earlier, BNP level is reduced in individuals with obesity, but BNP affects weight loss in turn, indicating the presence of a balance between the effect of obesity to reduce BNP level and the effect of BNP to improve obesity. As Inoue *et al.* reported earlier, BNP level was also inversely associated with insulin resistance in patients with heart failure [64]. Therefore, a similar balance may exist between the effect of insulin resistance to reduce BNP level and the effect of BNP to improve insulin resistance. In this regard, a subject with low BNP level is at a status in which the effect of obesity or insulin resistance is dominant over the effect of BNP to improve obesity or insulin resistance. Under this circumstance, the cardio-protective effects of BNP, such as natriuresis, diuresis, vasodilation, or lusitropy, could be compromised, making the subject prone to myocardial injury.

In addition to the obesity and insulin resistance, genetic factors should also be taken into consideration as factors for reduced BNP levels. Polymorphism in the

gene encoding BNP, natriuretic peptide precursor B (NPPB), has been reported to affect BNP expression levels [65]. As for polymorphisms in NPPB at T-381C, BNP level resulting from the genotype TT is lower than that resulting from the genotype TC or CC [64]. Seidelmann *et al*. reported that a polymorphism at the NPPB promoter, rs198389, was associated with the expression of NT-proBNP [66]. In that report, the authors showed that NT-proBNP expression, presumably BNP expression as well, was lower in subjects with the genotype AA at rs198389 than in subjects with the genotype AG or GG22. I, therefore, assume that low BNP levels result from a combination of factors including obesity, insulin resistance, and polymorphisms at NPPB and its promoter.

It is interesting that the slope of the curve shown in Fig. 6 looks biphasic; the slope is nearly horizontal when the BNP cut-off level is higher than 4 pg/mL, whereas the slope is precipitous at BNP cut-off levels lower than 4 pg/mL. This pattern may be reflective of a mechanism that compensates for the pathogenic effect of insufficient BNP level – a physiological mechanism that compensates for the compromised cardio-protective effect of the insufficient BNP level to attenuate injury to the cardiomyocytes, in a manner similar to the mechanism observed in the compensated status in patients with heart failure [67].

Regarding the subject with a high cTnI level (826.1 pg/mL) who was excluded from this study as mentioned in Materials and Methods, the BNP level was only 1.9 pg/mL. This subject may also be another example of a case with cTnI elevation under low BNP conditions.

One of the reasons why it was possible for us to identify the pathogenic effect of low BNP level was that I performed the assessment in the general population, rather than clinical patients. As mentioned earlier, it is known that BNP is expressed not only in patients with heart failure but also in patients with acute myocardial infarction [62]. Therefore, it would have been very difficult for us to identify subjects with BNP levels as low as those observed in the population in this study if I had chosen clinical patients with cardiac diseases as the study subjects. However, the putative mechanism by which a BNP level lower than that physiologically required exerts a pathogenic effect could be generalized to patients with cardiac diseases if, via the determination of influences of factors that suppress BNP expression (e.g., high BMI, insulin resistance, and genetic factors), I can assess whether BNP level in those patients are lower than physiologically required. If this becomes possible, the monitoring of BNP level would be greatly beneficial for patients with cardiac diseases as well as for the general population.

Table 1: Basic Characteristics of the Total Cohort.

	Unit	N = 2,001 (884 females and 1,117 males)
		Median (25%ile, 75%ile)
Age	years	56.0 (48.0, 63.0)
BMI	kg/m^2	22.6 (20.6, 24.8)
WC	kg/m^2	81.5 (75.7, 87.5)
SBP	mmHg	119.0 (109.0, 129.0)
DBP	mmHg	76.0 (68.0, 84.0)
Heart Rate	bpm	67.0 (61.0, 75.0)
Vital Capacity	L	3.4 (2.8, 4.1)
WBC	/μL	5200.0 (4300.0, 6100.0)
RBC	$10^4/\mu L$	462.0 (433.0, 491.0)
Hb	g/dL	14.2 (13.3, 15.2)
Ht	%	43.0 (40.5, 45.6)
PLT	$10^4/\mu L$	23.6 (20.6, 27.3)
ALB	g/dL	4.4 (4.2, 4.6)
AST	U/L	21.0 (18.0, 25.0)
ALT	U/L	19.0 (14.0, 26.0)
GGT	U/L	26.0 (17.0, 45.0)
UA	mg/dL	5.2 (4.3, 6.2)
eGFR	$mL/min/1.73\ m^2$	75.3 (67.5, 84.8)
BUN	mg/dL	13.0 (11.0, 16.0)
LDL-C	mg/dL	124.0 (106.0, 144.0)
HDL-C	mg/dL	62.0 (51.0, 75.0)
TG	mg/dL	88.0 (62.0, 130.0)
HbA1c	% (NGSP)	5.6 (5.4, 5.9)
Fasting Blood Glucose	mg/dL	98.0 (92.0, 106.0)
Framingham Risk Score		10.0 (7.0, 14.0)
BNP	pg/mL	5.6 (3.0, 10.3)
cTnI	pg/mL	1.5 (0.6, 2.8)
Current smoker	%	35.6
CKD	%	10.4
Dyslipidaemia	%	65.3
Hypertension	%	33.4
Diabetes	%	32.2

Table 2: Evaluation of the Quantifiable Range of the BNP Assay.

The samples were prepared by diluting Control L by dilution factors signified at the top row.

			Dilution factor						
N	Unit	Blank	64	32	16	8	4	2	Cont. L
1	pg/mL	0.0	0.9	1.5	3.3	6.3	14.6	28.6	60.7
2	pg/mL	0.0	0.8	1.9	3.0	6.7	14.0	27.4	53.8
3	pg/mL	0.0	1.0	1.8	3.3	6.5	14.2	26.6	55.2
4	pg/mL	0.0	0.8	1.5	3.2	6.2	13.5	27.1	52.6
5	pg/mL	0.0	0.7	1.6	3.1	6.4	13.3	28.2	55.3
6	pg/mL	0.0	0.7	1.5	3.2	6.8	13.3	27.2	52.3
7	pg/mL	0.0	0.7	1.3	3.3	6.4	13.1	28.4	56.2
8	pg/mL	0.0	0.7	1.6	3.5	6.7	13.0	25.9	49.8
9	pg/mL	0.0	0.6	1.7	3.2	6.3	13.3	27.3	54.3
10	pg/mL	0.0	0.6	1.6	3.1	6.6	13.0	26.2	52.1
Expected value (mean)	pg/mL	0.0	0.8	1.7	3.4	6.8	13.6	27.1	54.2
Measured value (mean)	pg/mL	0.0	0.8	1.6	3.2	6.5	13.5	27.3	54.2
Bias (%)	%	-	-10.3	-4.4	-4.8	-4.3	-0.3	0.7	0.0
CV%	%	-	16.7	11.4	3.9	3.0	4.1	3.4	5.4
Abs(bias)%+CV%	%	-	27.0	15.8	8.8	7.3	4.4	4.0	5.4

Table 3: Multivariate Analysis against Log(cTnI) in the Total Cohort and a Subgroup with BNP Level Lower than 4 pg/mL.

	Total Cohort				BNP Level Lower Than 4 pg/mL			
	Coefficient	SE	T	p-value	Coefficient	SE	t	p-value
Intercept	-2.698	0.221	-12.23	< 0.001	-2.647	0.529	-5.01	< 0.001
Log(BNP)	0.149	0.038	3.91	< 0.001	-0.423	0.130	-3.24	0.001
Gender	0.281	0.036	7.77	< 0.001	0.309	0.085	3.62	< 0.001
Age	0.020	0.002	12.91	< 0.001	0.021	0.004	5.69	< 0.001
BMI	0.015	0.005	3.23	0.001	0.008	0.010	0.76	0.446
SBP	0.003	0.001	3.67	< 0.001	0.005	0.002	2.03	0.043
Hb	0.015	0.013	1.13	0.260	0.015	0.032	0.46	0.647
eGFR	0.005	0.001	4.94	< 0.001	0.005	0.002	2.35	0.019

Table 4: Estimation with Structural Equation Model against Log(TnI) in the Group of Subjects with BNP Values Equal to or Less Than 1 pg/mL.

Clinical Factor			Estimate	SE	Test statistic	p-value	Standard regression coefficient
Log(cTnI)							
	←	Gender	0.408	0.256	1.597	0.110	0.408
	←	Age	0.018	0.010	1.701	0.089	0.018
	←	eGFR	0.003	0.005	0.501	0.616	0.003
	←	SBP	0.011	0.007	1.568	0.117	0.011
	←	Log(BNP)	-1.186	0.436	-2.718	0.007	-1.186
	←	BMI	0.022	0.026	0.825	0.409	0.022
	←	Hb	-0.176	0.087	-2.032	0.042	-0.176

Table 5: Estimation with Structural Equation Model against Log(TnI) in the Group of Subjects with BNP Values Equal to or Less Than 20 pg/mL.

Clinical Factor			Estimate	SE	Test statistic	p-value	Standard regression coefficient
Log(cTnI)							
	←	Gender	0.270	0.038	7.111	<0.001	0.270
	←	Age	0.021	0.002	12.549	<0.001	0.021
	←	eGFR	0.005	0.001	4.933	<0.001	0.005
	←	SBP	0.004	0.001	3.880	<0.001	0.004
	←	Log(BNP)	0.085	0.044	1.914	0.056	0.085
	←	BMI	0.012	0.005	2.470	0.014	0.012
	←	Hb	0.017	0.014	1.245	0.213	0.017

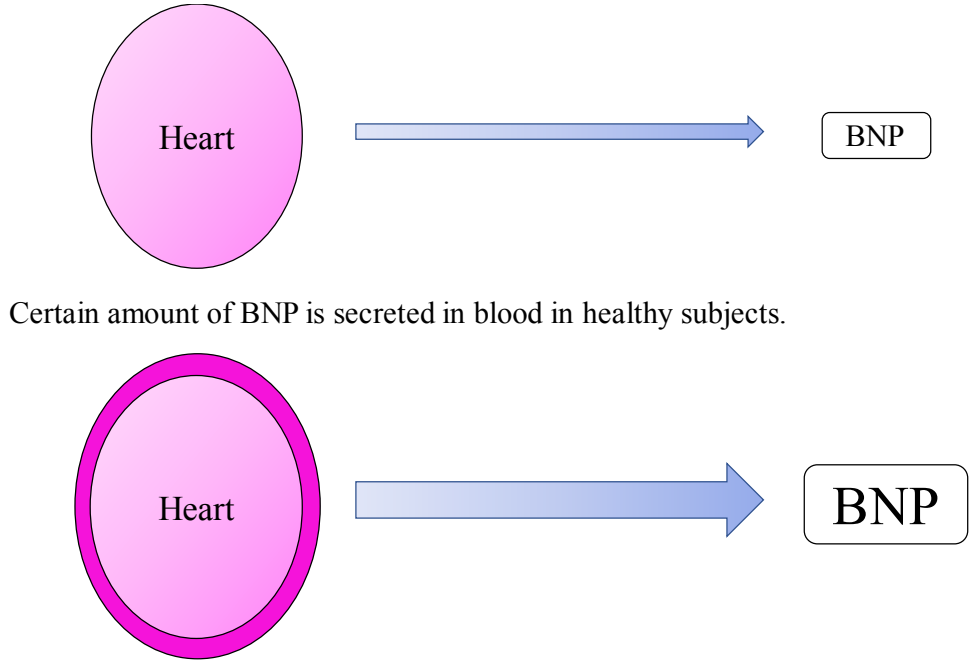

Fig. 1: BNP as a Biomarker for Heart Failure

Schematic diagrams to show why BNP can be used as a biomarker for heart failure by contrasting the amount of BNP secretion in healthy subjects (upper diagram) against subjects with heart failure (lower diagram).

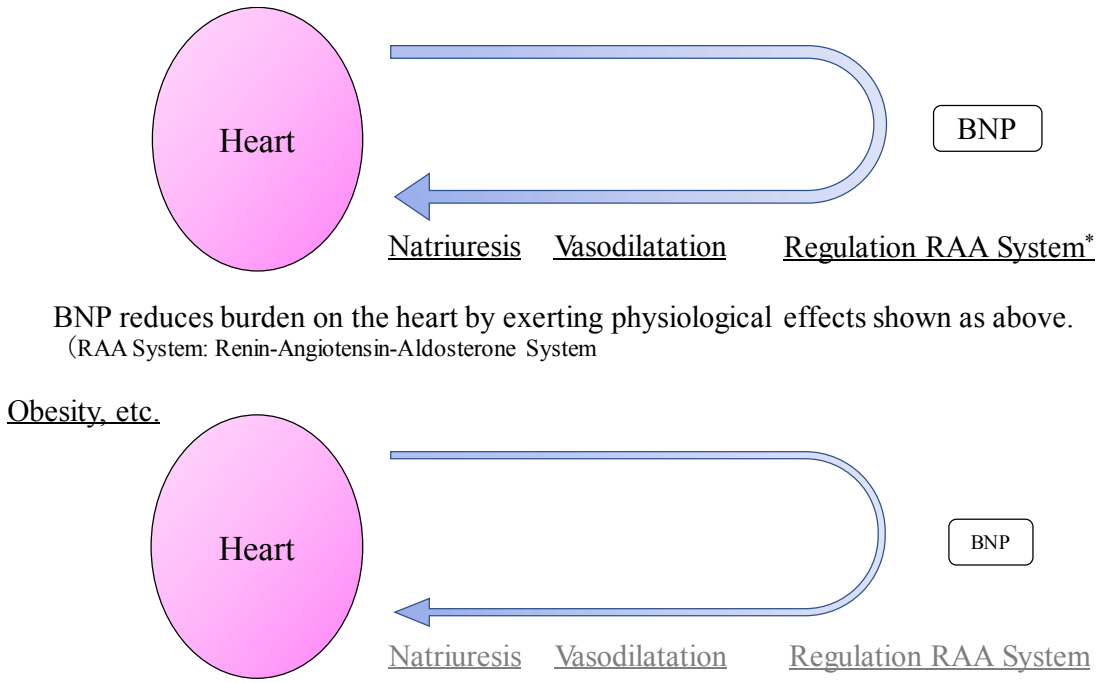

Fig. 2: BNP as a Physiologically Active Substance

Schematic diagrams to show functions of BNP when BNP is viewed as a physiologically active substance by contrasting functions in healthy subjects (upper diagram) against subjects with reduced BNP level such as by obesity (lower diagram).

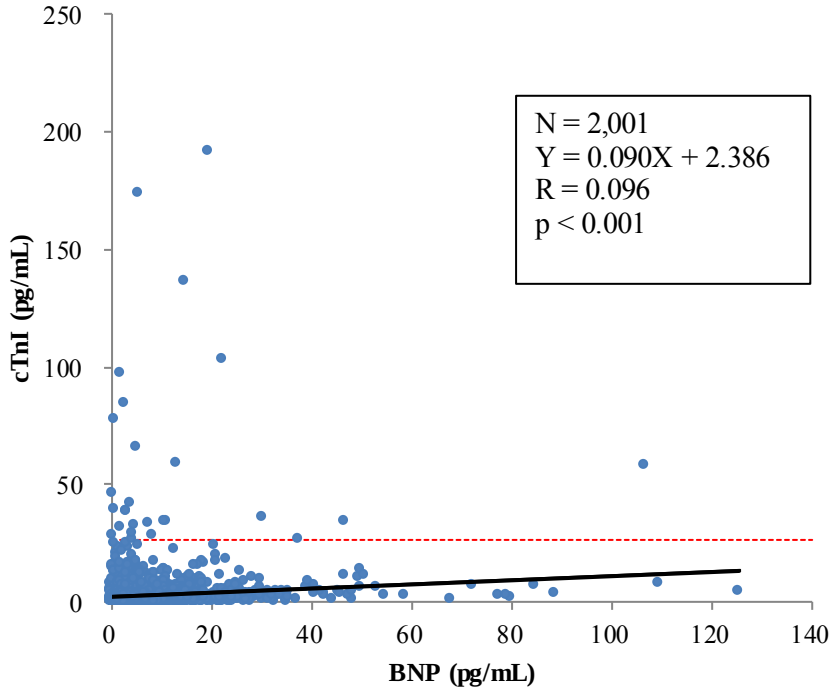

Fig. 3: Two-dimensional Plot of BNP (X-axis) and cTnI (Y-axis) Levels of the Total Cohort.

The solid line (——) signifies linear regression. The red dotted line (------) signifies the 99th percentile cut-off level of cTnI, 26.2 pg/mL.

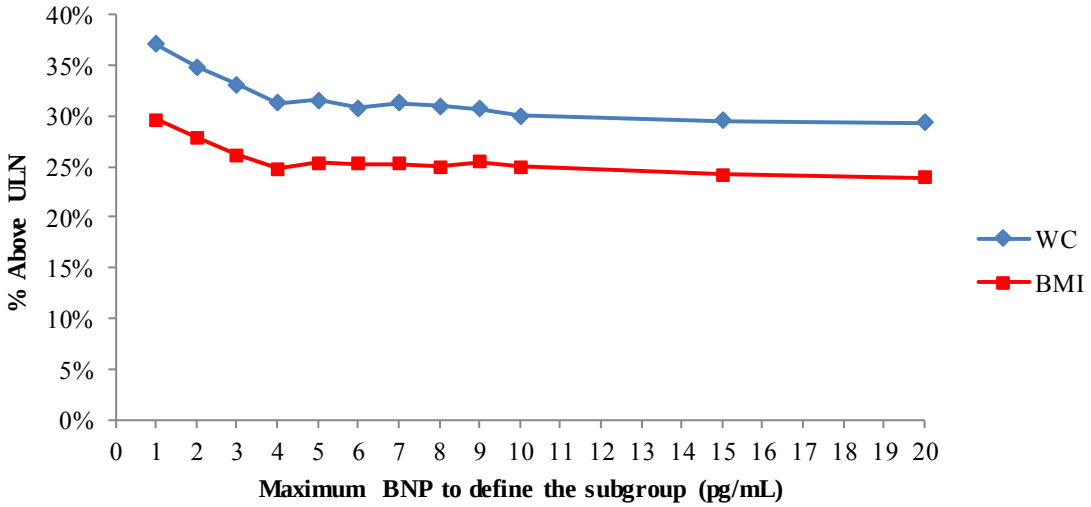

Fig. 4: Increased Percentage of Obesity in Low-BNP Subgroups.

Percentages of subjects exceeding the upper limit of normal (ULN) values of WC or BMI (Y-axis) in subgroups defined by the maximum BNP level shown in the X-axis.

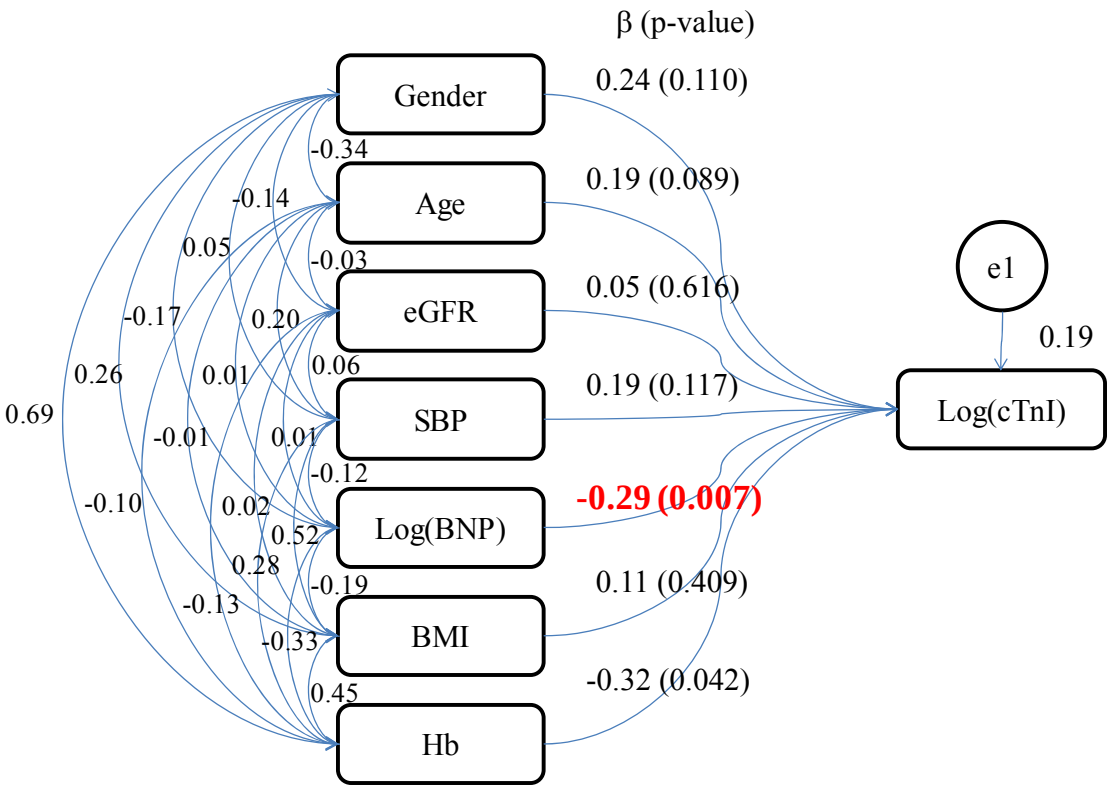

Fig. 5a: Path Diagram against Log(cTnI) in Subjects with BNP Levels Equal to or Lower Than 1 pg/mL (Low BNP Group, N = 81).

The coefficient of standardized regression (β) of each path is shown adjacent to the path. P-values are also shown in parentheses regarding the direct association between log(cTnI).

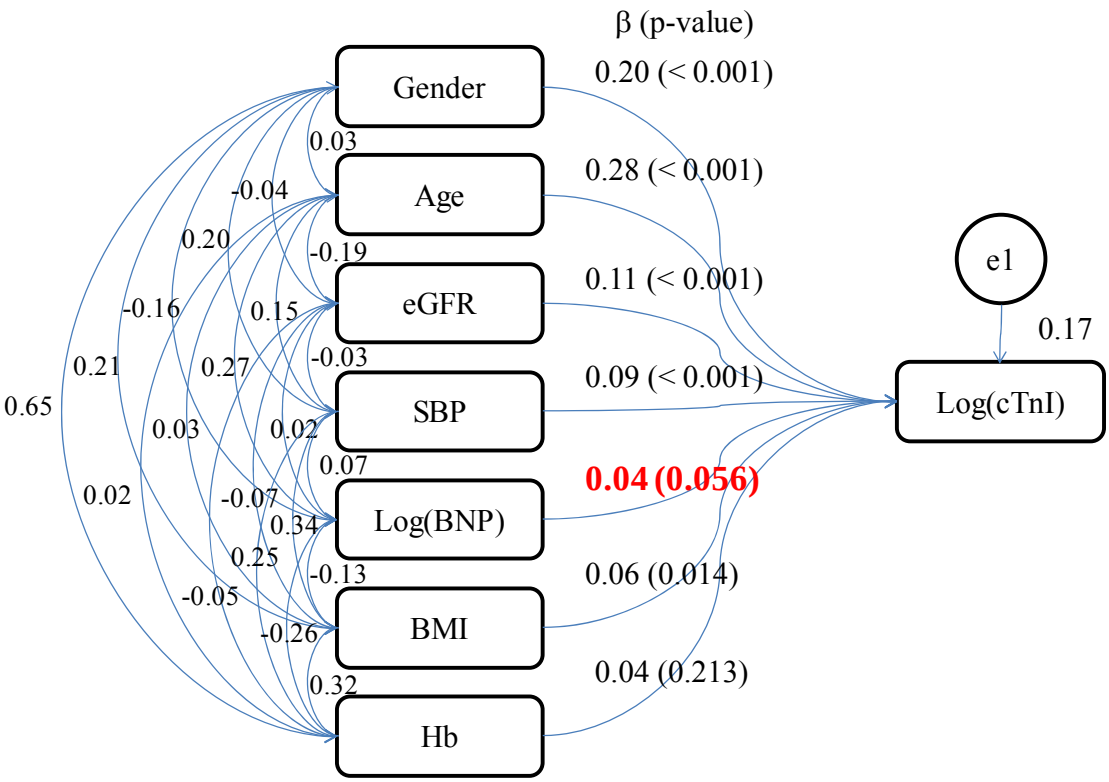

Fig. 5b: Path Diagram against Log(cTnI) for Subjects with BNP Levels Equal to or Lower Than 20 pg/mL (Control Group, N = 1,860).

The coefficient of standardized regression (β) of each path is shown adjacent to the path. P-values are also shown in parentheses regarding the direct association between log(cTnI).

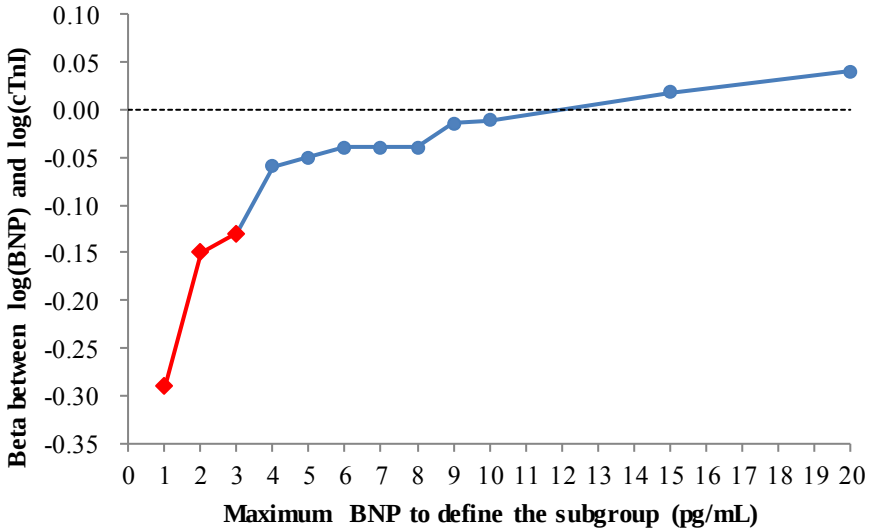

Fig. 6: Inversed Correlation Between between Log(BNP) and Log(cTnI) in Low-BNP Subgroups.

Betas between log(BNP) and log(cTnI) in Low-BNP subgroups defined by the maximum BNP levels were plotted in X-axis against the maximum BNP levels in Y-axis. The red diamonds (♦) signify beta values with p-values less than 0.005. The dotted line signifies level of beta equals zero.

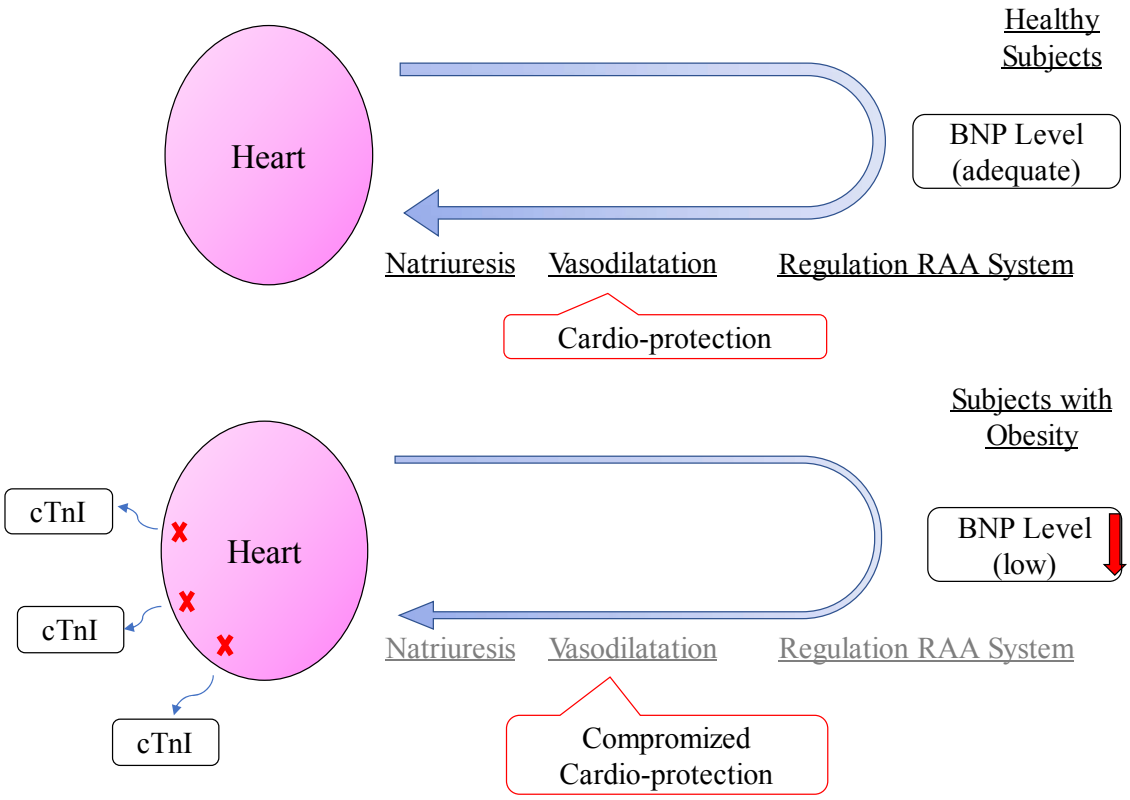

Fig. 7: Speculated Subsequence of Reduced BNP

From the result that BNP is inversely associated with cTnI, the cardio-protective effect by BNP is speculated to be compromised in subjects with suppressed BNP level such as by obesity (lower diagram) when contrasted with the cardio-protective effect exert by BNP in healthy subjects (upper diagram), which could bring about myocardial injury represented by the elevation of cTnI.

Chapter 4. Concluding Remarks

1. Summary

In Chapter 1, I evaluated the 13 biomarkers related with cardiovascular diseases in terms of their clinical specificity, utility for diagnosis, assessment of disease severity, monitoring of the treatment and assessment of prognosis. From this evaluation, BNP and cTnI resulted in the two best biomarkers (Chapter 1, Table 3). I, therefore, setup the objectives of this study,

1) to assess whether BNP or cTnI shows association with the risk factors for cardiovascular diseases,
2) to assess whether BNP and cTnI detect different populations and different (sub-clinical) cardiovascular diseases, and
3) to assess whether there is any positive or inverted association between BNP and cTnI.

In Chapter 2, I measured BNP and cTnI levels in 950 subjects (439 females and 511 males) who came to have annual health checkup to the checkup centers. Here, I have confirmed that BNP and cTnI detect populations with high cardiovascular risks in the general population and that BNP and cTnI detect

non-overlapping two different high-risk populations shown as the characteristic "L-shaped distribution" in the 2D plot (Chapter 2, Fig. 1).

In Chapter 3, I assessed with 2,001 general population (884 females and 1,117 males) whether elevated cTnI levels are more frequent in the subgroup with extremely low BNP as indicated by the "L-shaped distribution" by employing the covariance structure modeling analyses. The analyses showed significant inversed association between log(BNP) and log(cTnI) in the subgroups with the BNP levels lower than 4pg/mL (Chapter 3, Fig. 5a and 6). This result indicated that cardio-protective effect of BNP could be compromised at the extremely low BNP level (e.g. < 4 pg/mL) so that cTnI levels are more frequently elevated by the myocardial injury as the consequence.

Fig. 1 shows the consolidation of the results obtained in this study; quadrants A and C that are two independent populations high in cardiovascular disease risk confirmed in Chapter 2, and a population with extremely low BNP which has potential risk of cTnI elevation indicated in Chapter 3.

In conclusion, both BNP and cTnI are necessary for the screening of potential cardiovascular diseases because they detect different high-risk populations. Furthermore, there may be potential risk for the myocardial injury by the

compromised cardio-protective effect by BNP in the sub-population with extremely low BNP.

2. Limitations of this study

This study has the following limitations. Although the total numbers of subjects were high (950 in Chapter 2 and 2,001 in Chapter 3), the percentage of the subjects with cTnI level greater than the 99th percentile (26.2 pg/mL) was only 1.3% (27 subjects) and that of the subjects with BNP level greater than 40 pg/mL was only 1.5% (30 subjects), and thus, the bias in the population may have affected the results. The other limitation is that the range of the parameters of health status in this population was comparatively narrow; for example; the BMI was within 20.6 and 24.8 in 50% of the population. Therefore, slight deviations from the overall population (e.g., subjects with extremely high BMI) may have affected the results. Last but not least, although the conclusion that extremely low BNP level is one of the causes for the development of cardiovascular diseases is consistent with previous reports (e.g., obesity is associated with higher IHD risk; low BNP is associated with the incidence of IHD; elevated cTnI level is associated with cardiovascular risks in the general population), I do not have prospective and direct

evidence yet, which need to be obtained in the future.

3. Future prospective

To establish this screening method, I think it has to be validated from at least the following aspects;

1) the assessment of the prospective outcome with subjects exceeding cTnI cut-off who belong to quadrant A of Fig. 1 in Chapter 2 and subjects exceeding BNP cut-off who belong to quadrant C of Fig. 1 in Chapter 2,
2) the identification of pathogenic lesion in quadrants A and C,
3) the consensus on further medical examinations to be performed when a subject shows elevated cTnI or BNP,
4) the determination of optimal cut-off levels and frequency of this combination assay to be performed (e.g. once a year, once another year) for the prevention of cardiac sudden death, and
5) the cost-benefit analysis.

Once this screening system is established, the number of the test is expected to increase to reach, eventually, to the number of the total population above 40 years old, which is approximately 60 million in Japan. Assuming 1% of BNP-high and

1% of cTnI-high populations, there will be 1.2 million who will be subjected to further examinations and necessary treatments. Considering the annual cardiac sudden death in Japan, 30,000 to 40,000, I believe this screening would substantially reduce the number of the sudden deaths.

In this study, I selected BNP and cTnI as the two best cardiac biomarkers for the screening of potential cardiovascular diseases in the general population. Because these biomarkers are directly associated with the cardiac damages, the risk stage could be differentiated from the risk stage indicated by the conventional risk factors (e.g. hypertension, dyslipidemia, smoking, diabetes, CKD). Here I propose a concept of differentiated risk stages. The risk stage indicated by the elevation of either cTnI or BNP is considered as progressed when compared with the risk stage indicated by the presence of the conventional risk factors because the conventional risk factors are the factors that could cause myocardial damages in the future whereas the elevation of cTnI or BNP indicates that myocardial damages are present (Fig. 2).

In a rapidly aging society like Japan, I believe early detection of diseases for the early intervention would greatly help extending the healthy life activating the society by the increased the ratio of productive population.

The concept of differentiated risk stages could be applied not only to the cardiovascular diseases but also to the oncology area, for example. As shown in Fig. 3, factors such as smoking, western diet, obesity, toxic chemicals, insult to immune function could be the indicators for the early risk stage, while transformation of cell phenotypes or functions such as constitutive signaling for cell growth, anti-apoptotic ability, unresponsiveness to cell-to-cell contact inhibition, ability to inactivate immunity, secretion of growth factors, or ability for cell adhesion and infiltration are considered to be the indicators for the advanced risk stage. Human epididymis protein 4, or HE4, could be the candidate biomarker to indicate the advanced risk stage because HE4 has been shown to enhance tumor growth and promote cell adhesion and infiltration [68, 69].

4. Acknowledgement

I would like to express my deep gratitude to all those who provided me with the instructions, supports and encouragement during the preparation of this dissertation. Especially, I would like to thank Professor Akiyoshi Fukamizu for all his instructions and considerations throughout my dissertation writing. I am also indebted to Dr. Izuru Masuda, Dr. Kiminori Kato and Dr. Michihiro Yoshimura for

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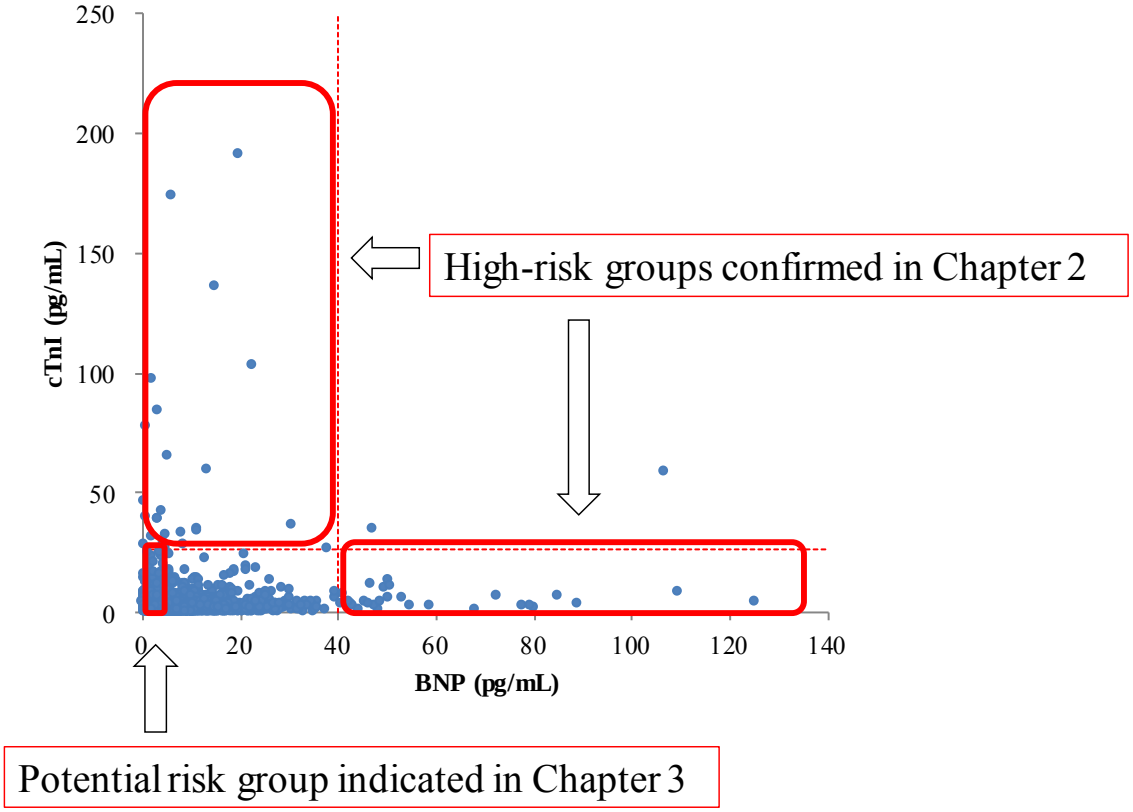

Fig. 1: Summary of High Risk Groups

Schematic diagram to show the groups of high cardiovascular risks indicated in Chapter 2 and Chapter 3. In Chapter 2, the group with cTnI level above the cut-off 26.2 pg/mL and the group with BNP level above the cut-off 40 pg/mL showed significantly high FRS and CTR. In Chapter 3, the group with BNP level less than 4 pg/mL showed inversed association with cTnI, indicating a potential for cTnI elevation.

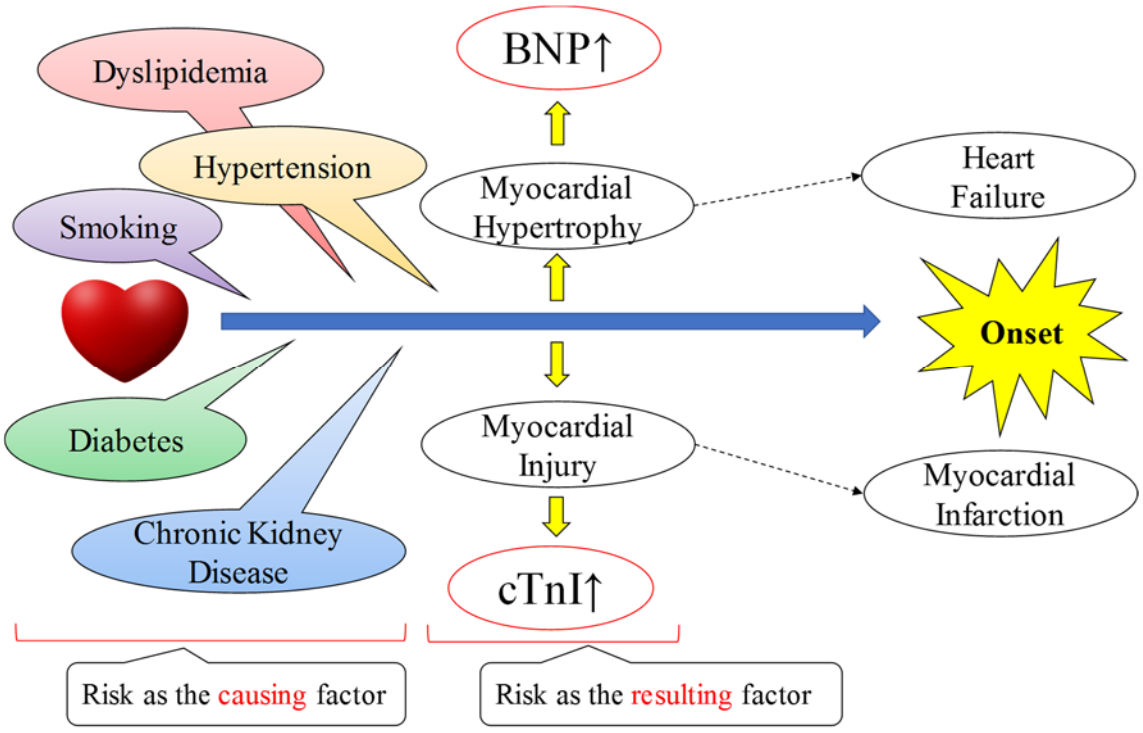

Fig. 2: Schematic Diagram of the Concept of Risk Stage in the Case of Cardiovascular Diseases

Schematic diagram to show differentiating stages of risks; a risk as a causing factor and a risk as a resulting factor. The former risk includes smoking, hypertension, dyslipidemia, diabetes and chronic kidney disease. The latter risk includes myocardial hypertrophy where BNP is the indicator and myocardial injury where cTnI is the indicator.

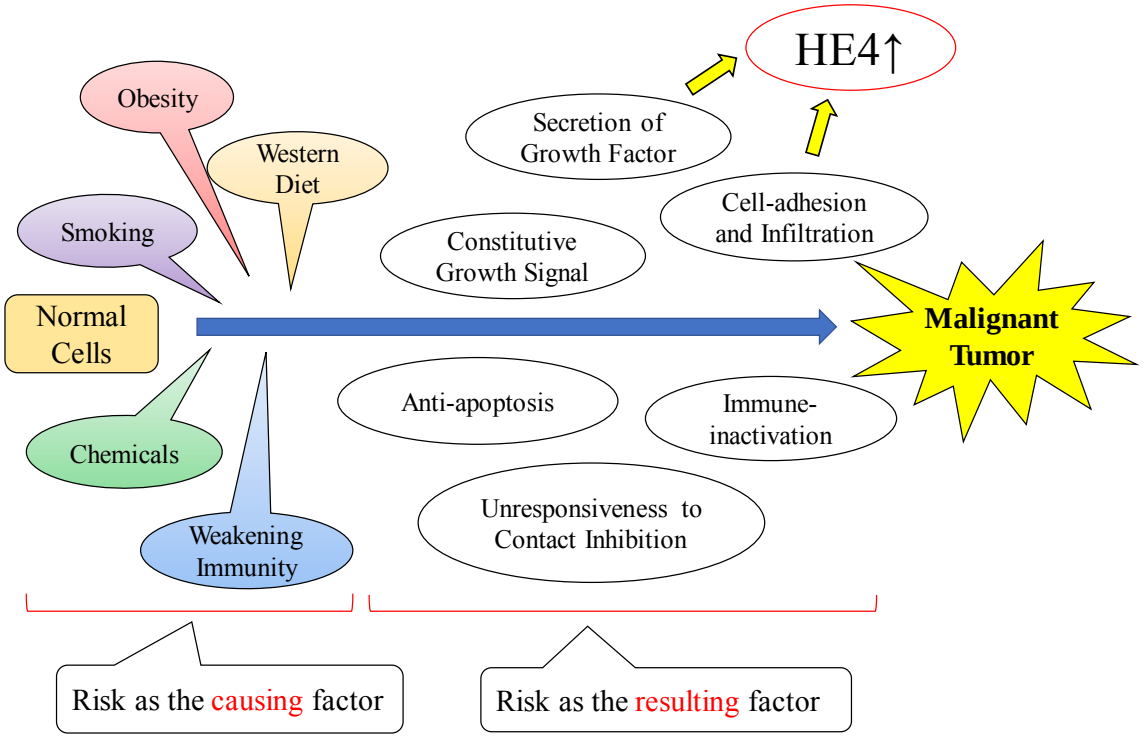

Fig. 3: Schematic Diagram of the Concept of Risk Stage in the Case of Malignant Tumor Development

Schematic diagram to show differentiating stages of risks; a risk as a causing factor and a risk as a resulting factor. The former risk includes obesity, western-style diet, toxic chemicals and weakening immunity. The latter risk includes constitutive growth signal, anti-apoptosis, unresponsiveness to cell-to-cell contact inhibition, immune-inactivation, secretion of growth factors and enhanced ability of cell adhesion and infiltration. Human epididymis protein 4, or HE4, is deemed as the candidate biomarker as the indicator for tumor growth factor and enhanced ability of cell adhesion and infiltration.

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