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Urocortin2 measurement for heart failure assessment

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Despite the efficacy of many therapies for heart failure, it remains a leading cause of morbidity and mortality worldwide, with many patients progressing to advanced stages of the condition. Since the standard treatment for heart failure includes small-molecule drugs targeting G protein-coupled receptors (GPCRs), GPCRs are still considered novel targets for the diagnosis and treatment of cardiovascular diseases. Corticotropin-releasing hormone receptor 2, a highly expressed GPCR in cardiomyocytes, and its ligand, urocortin2 (UCN2), have been reportedly associated with cardiovascular diseases; however, their clinical significance remains unclear. In this study, a UCN2 measurement assay was developed to measure blood UCN2 levels in patients with heart failure. The assay showed that blood UCN2 values indicated a negative relationship with cardiac ejection fraction in 52 patients with heart failure. Blood UCN2 levels were not correlated with brain natriuretic peptide, a standard marker of heart failure, and were higher in patients with cardiomyopathy than in those with heart failure, suggesting that measuring blood UCN2 levels may be a novel test for assessing the pathophysiology of heart failure.

Keywords Heart failure, UCN2, ELISA assay, CRHR2, GPCR, Peptide

Heart failure is a clinical syndrome characterized by the inability of the heart to meet the circulatory demands of the body at rest or during stress. It remains a leading and increasing cause of morbidity and mortality worldwide despite the efficacy of many therapies for patients with heart failure[1,2]. Heart failure symptoms, such as fatigue and dyspnea, are among the main predictors of poor patient outcomes[2,3]. In addition to the symptoms, biomarkers are largely used in the diagnosis, management, and prognosis of heart failure[1]. For example, plasma concentrations of brain natriuretic peptide (BNP) or its N-terminal prohormone of BNP (NT-proBNP) are a mainstay for the diagnosis of heart failure. The natriuretic peptides BNP and NT-proBNP have been shown to have excellent sensitivity for the diagnosis of heart failure. However, because of their low specificity, these biomarkers must be assessed in the context of other diagnoses and patient characteristics that affect the diagnostic thresholds. Measuring other circulating plasma proteins may aid our understanding of heart failure-specific signaling, thereby supporting new therapeutic and diagnostic efforts for chronic heart failure[4].

G protein-coupled receptors (GPCRs) are involved in numerous physiological and pathological processes and thus serve as important drug targets in cardiovascular diseases[5]. Small-molecule drugs targeting GPCRs, particularly β-adrenoceptor and angiotensin II receptor antagonists, are the standard treatments for heart failure[6]. Despite maximal therapy, many patients with heart failure progress to advanced heart failure with persistent symptoms[7], suggesting that additional uncharacterized GPCRs may also mediate disease pathophysiology. Among GPCRs whose relationship with cardiovascular diseases is unknown, we have shown that corticotropin-releasing hormone receptor 2 (CRHR2) is highly expressed in cardiomyocytes, and cardiomyocyte-specific deletion of CRHR2 protected mice from pressure overload-induced cardiac dysfunction[8]. We also developed a novel, oral, small-molecule antagonist of CRHR2 and demonstrated its ability to prevent cardiac dysfunction in murine models[9]. CRHR2 belongs to the secretion-like class B family of GPCR, a peptide receptor that binds physiologically important peptide hormones to transduce downstream cellular signals[10]. A review of pharmacological studies has shown that different peptides display different binding affinities for CRHR2 and that urocortin 2 (UCN2) and UCN3 are CRHR2-selective ligands[11]. A high concentration of UCN2 therapy

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using an intravenous injection of a viral vector system increases basal cardiac systolic function in mice with decreased blood pressure[12]. The effects of chronically elevated UCN2 levels in murine heart failure models are controversial. Although chronic UCN2 treatment with a decrease in blood pressure reduced infarct size in a murine myocardial infarction model[13], chronic UCN2 activation without vasorelaxation may have cardiotoxic effects[8]. These basic research findings suggest that UCN2 is associated with heart failure. However, the clinical significance of UCN2 in heart failure remains unclear.

In this study, we aimed to develop a human UCN2-specific enzyme-linked immunosorbent assay (ELISA) assay and investigate blood UCN2 concentrations in patients with heart failure. These findings are expected to provide novel research insights on the application of CRHR2 and UCN2 in clinical practice.

Results

Establishing a human UCN2-specific ELISA assay

We prepared an antibody against human UCN2 (1–41 aa), which detects synthetic human UCN2, but not synthetic human UCN1 nor UCN3 (Fig. 1a). A solid-phase sandwich ELISA assay for human UCN2 was developed, using the same antibody against human UCN2 for antigen capture and detection. The working range of this ELISA was 70–4500 pg/mL when synthetic human UCN2 in dilution buffer was used as a calibrator (Fig. 1b). The analytical limit of quantification of this assay was determined according to the guidelines provided by the Clinical and Laboratory Standards Institute. The sensitivity limit of the UCN2-specific ELISA assay was 17.9 pg/mL. The specificity of the ELISA assay was tested by adding an anti-UCN2 antibody in molar excess to synthetic human UCN2, diluted in a dilution buffer (Fig. 1c). Excess anti-UCN2 antibody blocked the ability to detect synthetic human UCN2. We also evaluated the potential interference with UCN2 measurements using free bilirubin F and C, hemoglobin, and chyle. Bilirubin F (up to 10 mg/dL) and C (up to 20 mg/dL), hemoglobin (up to 245 mg/dL), and chyle (up to 1650 FTU) did not significantly affect the accuracy of the ELISA. Dilution tests were performed on human samples obtained from two different matrices (serum and

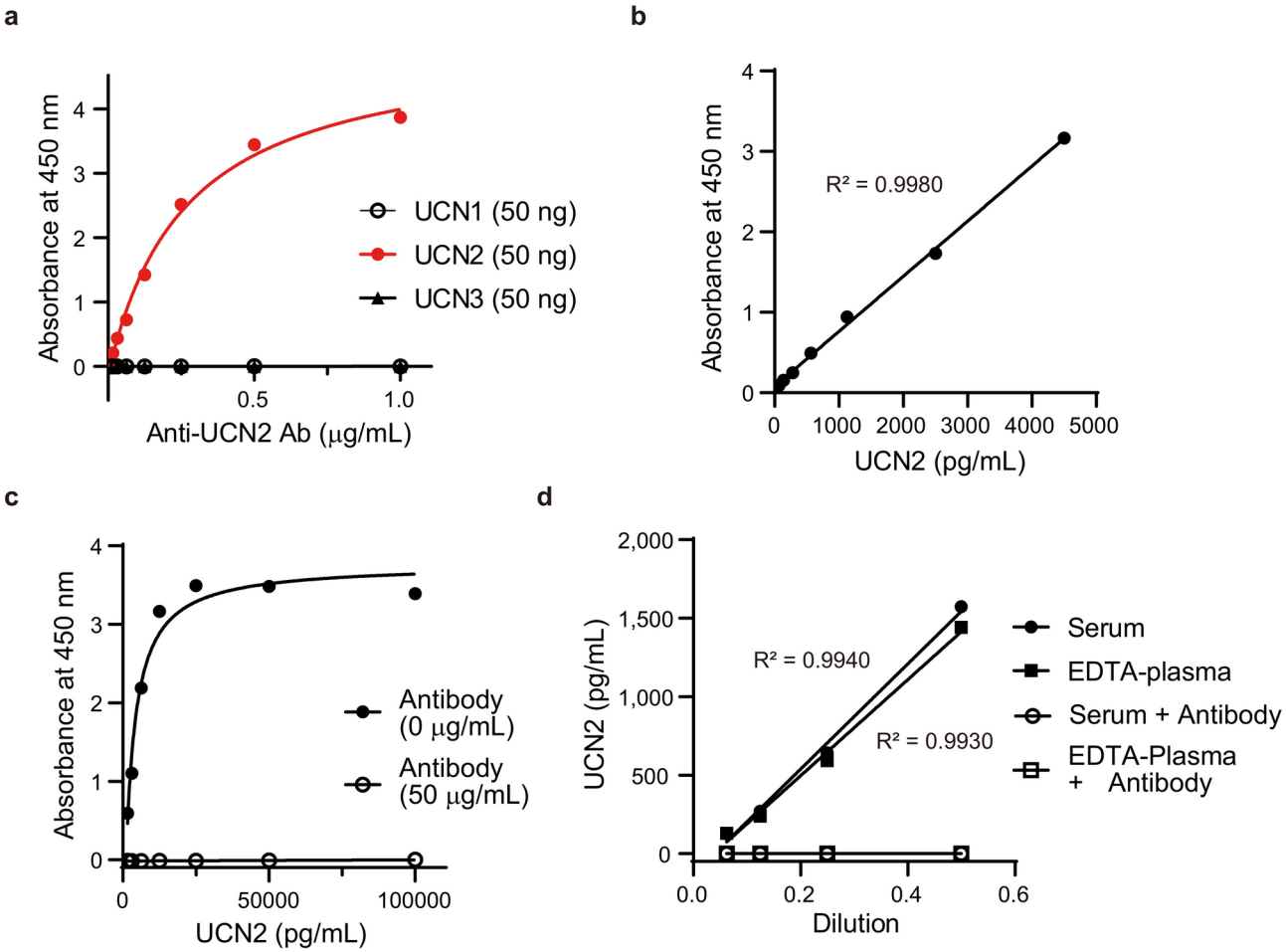

Fig. 1. Human UCN2 enzyme-linked immunosorbent assay (ELISA). (**a**) Antibody against human UCN2 detects synthetic human UCN2, but not synthetic human UCN1 or UCN3. (**b**) Linearity tests of human UCN2 were performed. (**c**) The specificity of the ELISA assay was tested by adding an anti-UCN2 antibody. (**d**) UCN2 dilution linearity comparison in human serum or ethylenediaminetetraacetic acid (EDTA) plasma samples. Relationships between parameters were evaluated using Pearson's correlation coefficient of determination (R^2).

ethylenediaminetetraacetic acid [EDTA] plasma). The curve produced by serially diluted serum samples was parallel to that produced by diluted heparin and citrate plasma. Excess anti-UCN2 antibody blocked the ability to detect human UCN2 in the serum and plasma, indicating that this assay system specifically determined the concentration of UCN2 in the serum and plasma (Fig. 1d).

A recovery test was performed by adding a human UCN2 standard (562.5–2250 pg/mL) in human serum and EDTA plasma (Fig. 2a). The recovery rate was between 91.1 and 106.2% of the original concentration for serum and between 83.8 and 86.1% of the original concentration for EDTA plasma. Interpretations of intra-variations in the assay were performed using three quality control samples (low, medium, and high) (Fig. 2b). The coefficients of variation (CVs) of intra-assay imprecision were 1.5, 3.1, and 1.2 in the low, medium, and high controls, respectively. Inter-variations in the assay were interpreted using three quality control samples (Fig. 2c). In the low, medium, and high controls, the inter-assay CV values were 2.5, 3.8, and 5.4, respectively.

Measurement of human UCN2 levels in patients with heart failure

To examine the relationship between UCN2 levels and parameters in patients with heart failure, we measured serum UCN2 levels in 52 patients with heart failure. The patient characteristics, demographic data, and laboratory parameters are summarized in Supplementary Table S1. The distribution of serum UCN2 levels in the study population is shown in Fig. 3a. The asymmetrical distribution of UCN2 led us to consider log-transformation for the statistical analyses, assuming a normal distribution for $\log_{10}$ UCN2. The median UCN2 log concentration was 2.61 (range 1.44–4.28; interquartile range [IQR] 2.04–2.86). None of the patients had UCN2 levels below the detection limit of the assay.

To investigate the clinical significance of serum UCN2 levels, the patients were dichotomized according to the median $\log_{10}$ UCN2 (Table 1). No significant differences were observed between the groups in terms of sex, body mass index, medication, or laboratory parameters, including estimated glomerular filtration rate and troponin. When comparing the high and low median groups, patients with high UCN2 levels had lower left ventricular ejection fraction (30.5% vs 41.5%) and systolic and diastolic blood pressure (95 vs 111 mmHg: 56.5 vs 61.5 mmHg) than those with low UCN2 levels. Log_{10} UCN2 showed low correlations with left ventricular ejection fraction, age, and systolic and diastolic blood pressure (Supplementary Table S2).

A trend was observed that the higher the BNP, a known biomarker for predicting cardiac dysfunction, the lower the ejection fraction (Fig. 3b). High UCN2 levels were significantly correlated with a decrease in ejection fraction (Fig. 3c), suggesting that high UCN2 levels are associated with left ventricular systolic dysfunction. Moreover, there was a non-significant correlation between UCN2 and BNP levels (Fig. 3d). UCN2 levels may represent the pathological condition of patients with heart failure, which differs from the condition indicated by BNP levels.

Common causes of heart failure include arrhythmias, coronary artery diseases, valvular heart diseases, cardiomyopathies, and infiltrative diseases[6,14]. To examine the relationship between UCN2 and the causes of heart failure, UCN2 and BNP levels in patients with each cause of heart failure are shown in Fig. 3e,f. Although BNP values were similar among patients with all causes of heart failure, UCN2 values increased in patients with cardiomyopathy (Fig. 3g). Advanced heart failure is indicated by severe symptoms, cardiac dysfunction (left

a

Analyte	Spiked amount (pg/mL)	Expected concentration in spiked sample (pg/mL)	Recovery (%)
Human serum (x2)	2,250	2,250	91.1
	1,125	1,125	101.4
	562.5	562.5	106.2
Human EDTA plasma (x2)	2,250	2,250	85.1
	1,125	1,125	83.8
	562.5	562.5	86.1

b

UCN2 concentration (pg/mL)	Intra-assay variation (n = 4)	
	SD (pg/mL)	CV (%)
2,078.5	25.2	1.2
503.7	15.6	3.1
165.5	2.5	1.5

c

UCN2 concentration (pg/mL)	Inter-assay variation (n = 4)	
	SD (pg/mL)	CV (%)
2,211.7	119.4	5.4
534.4	20.5	3.8
171.8	4.2	2.5

Fig. 2. Distribution of serum UCN2 concentration. (**a**) The recovery test was performed by adding a human UCN2 standard (562.5–2250 pg/mL) to human serum or ethylenediaminetetraacetic acid (EDTA) plasma. (**b**, **c**) Intra-assay variations (**b**) and inter-assay variations (**c**) were performed using four quality control samples. UCN2, urocortin 2.

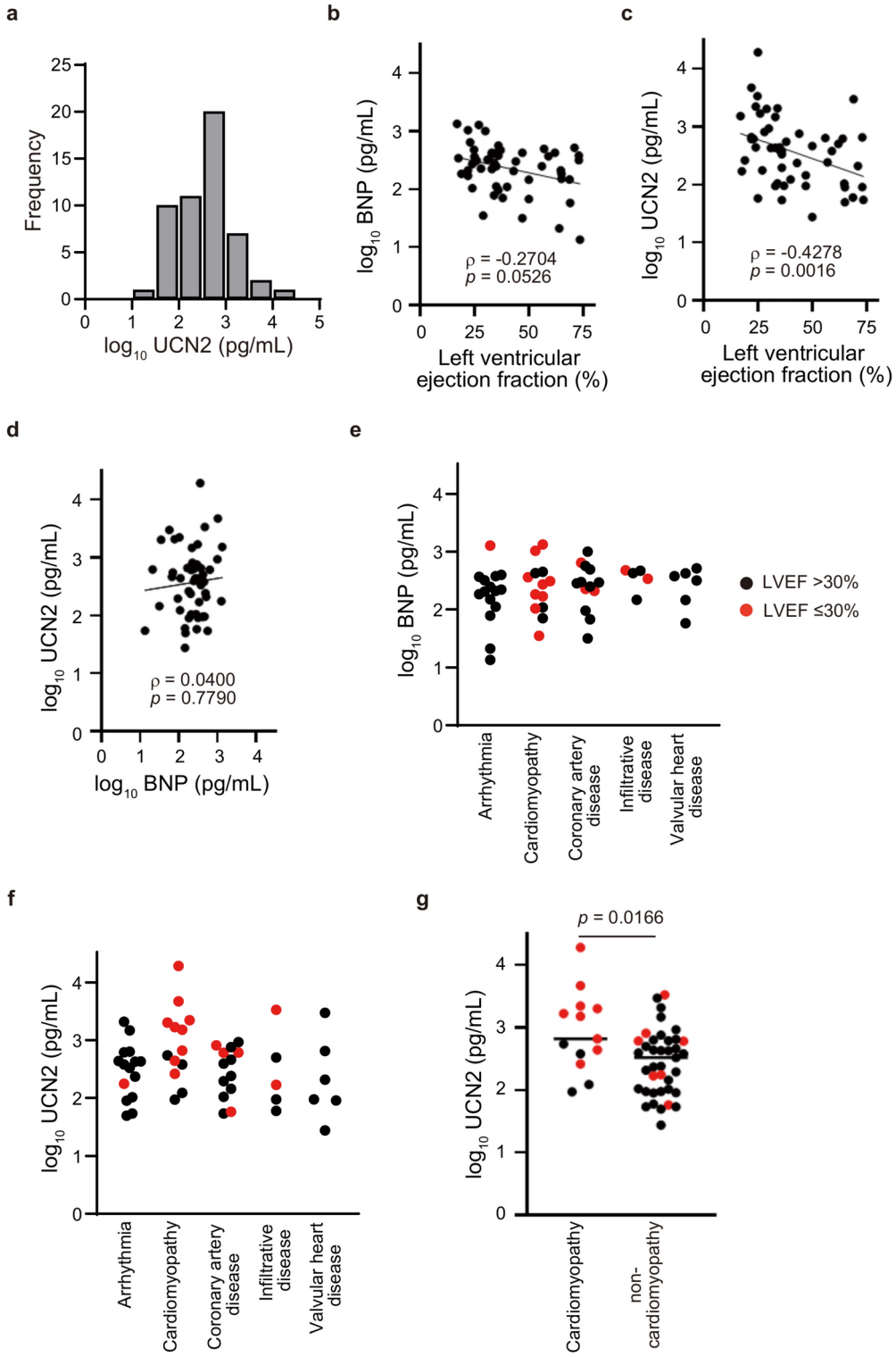

Fig. 3. Correlation between UCN2 and left ventricular ejection fraction. (**a**) Distribution in serum UCN2 concentration (log_{10} UCN2) in patients with heart failure ($n = 52$). (**b**) Correlations between LVEF and log_{10} brain natriuretic peptide (BNP). (**c**) Correlations between LVEF and log_{10} UCN2. (**d**) Correlations between log_{10} BNP and log_{10} UCN2. (**e**–**g**) BNP and UCN2 levels in various cardiac diseases. Spearman correlation analysis was performed (**b**–**d**): ρ, Spearman correlation coefficient. Data are represented as median and analyzed using the Mann–Whitney U test (**g**). UCN2, urocortin 2; LVEF, left ventricular ejection fraction.

	Log UCN2 > median High ($n=26$)	Log UCN2 < median Low ($n=26$)	
Age (years), mean ± SD	68.5 ± 17.5	76 ± 10.1	$p=0.18$
Gender: male/female, *n* (%)	19/7 (73.1%)	17/9 (65.4%)	$p=0.55$
BMI (kg/m^2), mean ± SD	22.1 ± 5.2	21.6 ± 3.5	$p=0.92$
Systolic blood pressure (mmHg), median (IQR)	95 (84–100.3)	111 (92.3–120)	$p=0.02$
Diastolic blood pressure (mmHg), median (IQR)	56.5 (52.5–63)	61.5 (57–73.5)	$p=0.04$
Heart rate (bpm), mean ± SD	73.0 ± 12.0	70.7 ± 11.8	$p=0.49$
Laboratory parameters			
WBC ($\times 10^3$/μL), median (IQR)	5800 (4650–7050)	5300 (4250–6175)	$p=0.41$
Hb (g/dL), median (IQR)	11.75 (10.5–13.63)	11.25 (10.65–13.4)	$p=0.46$
BUN (mg/dL), median (IQR)	21.8 (16.95–29)	28.25 (16.25–43.08)	$p=0.39$
Creatinine (mg/dL), median (IQR)	1.06 (0.98–1.42)	1.11 (0.98–1.66)	$p=0.69$
eGFR (JSN-CKDI, mL/min/1.73 m^2), mean ± SD	50.5 ± 18.6	43.1 ± 18.2	$p=0.16$
Uric acid (mg/dL), median (IQR)	6.7 (5.85–7.75)	6.7 (5.5–8.9)	$p=0.96$
HbA1c (%), median (IQR)	6.15 (5.3–6.75)	6.1 (5.85–6.53)	$p=0.84$
Log_{10} UCN2, median (IQR)	2.85 (2.73–3.31)	2.05 (1.91–2.37)	
Log_{10} BNP, median (IQR)	2.41 (1.98–2.61)	2.46 (2.17–2.63)	$p=0.72$
TroponinT (ng/mL), median (IQR)	0.04 (0.02–0.06)	0.04 (0.02–0.09)	$p=0.84$
CRP (mg/L), median (IQR)	0.17 (0.11–1.86)	0.25 (0.09–0.84)	$p=0.82$
Echocardiography			
LVDd (mm), median (IQR)	54 (45.5–64.5)	49.5 (43–57.3)	$p=0.39$
LVDs (mm), mean ± SD	45.6 ± 13.8	38.6 ± 15.2	$p=0.09$
LAD (mm), mean ± SD	46.5 ± 10.5	46 ± 7.4	$p=0.85$
LVEF (%), median (IQR)	30.5 (24–45.5)	41.5 (34.75–64.78)	$p=0.013$
E/e' ratio, median (IQR)	17.86 (13.5–29.77)	18.72 (15.18–25.14)	$p=0.80$
TRPG (mmHg), median (IQR)	27 (23–36.8)	34 (24–43.85)	$p=0.27$
Medication			
β blocker, *n* (%)	21 (80.8%)	17 (65.4%)	$p=0.21$
ACEi/ARB, *n* (%)	19 (73.1%)	15 (57.7%)	$p=0.24$
MRA, *n* (%)	20 (76.9%)	14 (53.8%)	$p=0.08$
ARNI, *n* (%)	0 (0%)	0 (0%)	
SGLT2i, *n* (%)	2 (7.7%)	2 (7.7%)	$p=1.0$
Diuretics, *n* (%)	20 (76.9%)	23 (88.5%)	$p=0.27$
Furosemide (mg), median (IQR)	20 (15–40)	20 (10–40)	$p=0.46$
Tolvaptan (mg), median (IQR)	0 (0–4.69)	0 (0–7.5)	$p=0.47$

Table 1. Comparison between patients' characteristics in relation to log_{10} UCN2.

ventricular ejection fraction ≤ 30%), and impaired exercise capacity[15]. Among patients with cardiomyopathy, UCN2 levels tended to be particularly high in those with low left ventricular ejection fraction.

Discussion

Although patients with chronic heart failure have improved outcomes following the implementation of evidence-based therapies, they continue to progress to advanced stages of the disease[15]. Higher BNP levels are associated with a greater risk of adverse short- and long-term outcomes of heart failure. However, measuring the severity of heart failure using BNP alone is not always sufficient[16]. It is also difficult to identify the cause of heart failure using only BNP[17]. New biomarkers may allow better prognostic stratification and assessment of the mechanisms of disease progression. Several studies have reported that UCN2 is a novel biomarker of cardiovascular diseases[18,19]. Therefore, in this study, we developed a UCN2-specific ELISA assay and examined the potential clinical significance of UCN2 measurements. ELISA showed that blood UCN2 concentrations indicate a low correlation with a decrease in the left ventricular ejection fraction in patients with heart failure. Additionally, blood UCN2 concentrations did not correlate with blood BNP concentration, suggesting that the blood UCN2 concentration indicates a different pathology of heart failure than blood BNP concentration. Although the number of cases was limited, BNP levels were elevated in a range of heart diseases. In contrast, UCN2 levels were elevated in cardiomyopathies, especially in patients with severe cardiac dysfunction (left ventricular ejection fraction ≤ 30%), and may be involved in the pathology of cardiomyopathies.

BNP and UCN2 differ in that BNP is secreted from the heart, whereas UCN2 acts on the heart. BNP secreted from cardiomyocytes in response to myocardial stretching in heart failure is believed to be a protective hormonal response to mechanical stress in the heart through the regulation of blood pressure and body fluid volume via the kidneys[20–22]. Since CRHR2, which UCN2 binds to, is expressed in cardiomyocytes, CRHR2 activates

Gs-mediated adenylyl cyclase, resulting in increased intracellular cAMP concentration[23,24]. CRHR2 deficiency decreases cardiac contraction accelerated by acute intravenous injection of UCN[25]. However, continuous overexpression of Gs in the heart results in murine cardiac dysfunction[26]. In addition to diagnosing heart failure, several biomarkers such as troponin have been used to determine the degree of myocardial injury[27]. However, we found no difference in troponin values between the high and low UCN2 median groups (Table 1), suggesting that UCN2 values do not directly reflect myocardial damage. It remains unclear which organs and mechanisms secrete UCN2. Further studies are required to examine the clinical significance of UCN2 expression in heart failure.

Our findings revealed that UCN2 levels were associated with age and blood pressure, as well as left ventricular ejection fraction. Intravenous administration of UCN2 reduced basal mean arterial pressure in rats[23]. In this study, patients with high UCN2 levels had severe cardiomyopathy. Hypotension has been reported in patients with heart failure and is associated with high mortality rates[28]. Individuals with dilated cardiomyopathy tend to be younger than those with other etiologies of heart failure[29]. The inclusion of cardiomyopathy with cardiac systolic dysfunction may contribute to the analysis of UCN2 expression with respect to age and blood pressure. Therefore, it is necessary to clarify the relationship between UCN2 and cardiac diseases by studying a larger number of patients.

A limitation of this study is that UCN2 levels were measured in patients with heart failure, but not in healthy individuals. Therefore, it is necessary for future studies to clarify the relationship between UCN2 and cardiac diseases in both healthy individuals and patients with cardiovascular illnesses. In addition, the human UCN2-specific ELISA assay developed in this study has not been directly compared with other UCN2 assays. The characteristics of this assay may be further clarified by comparison with other assays.

In this study, we developed a human UCN2-specific ELISA assay to investigate blood UCN2 concentrations in patients with heart failure. High UCN2 levels were significantly correlated with a decrease in ejection fraction in these patients. These results suggest that measuring UCN2 levels may be relevant to the pathological conditions of patients with heart failure. Future studies using the UCN2 levels are expected to identify unique biomarkers for cardiovascular diseases.

Methods

Monoclonal antibodies against human UCN2

Wistar rats were immunized with the human UCN2 peptide obtained from RaQualia Pharma Inc. (Nagoya, Japan). Antibody titters in the plasma of the immunized rats were monitored using ELISA, and hybridomas were generated by fusing splenocytes with X63 myeloma cells. After selecting the hybridomas with azaserine and hypoxanthine, samples of the medium were screened for UCN2 antibodies using ELISA. Seventeen hybridomas were expanded and sub-cloned. The four monoclonal antibodies (mAbs) were isotyped using a commercial kit (BD Biosciences, Tokyo, Japan). The hybridomas were adapted to a serum-free medium, and mAbs were purified from the hybridoma culture medium on a protein G–agarose column. Antibodies were eluted from the protein G column with glycine·HCl (pH 2.5). The same antibody was used to establish the UCN2 ELISA format.

UCN2 ELISA assay

After blood samples (100 µL) were diluted with 100 µL of dilution buffer (1% Bovine Serum albumin [BSA], 0.05% Tween20, 5 g/L Emulgen 109P, 0.5 M NaCl, 50 mg/mL murine IgG, and 0.05% ProClin300 in phosphate-buffered saline [PBS]), the samples were placed into a 96-well plate coated with anti-UCN2 antibody and incubated at 25 °C for 12 h. After incubation, the plate was washed four times with washing buffer (0.05% Tween20 in 10 mM phosphate-buffer). The plate was added with the second HRP-labeled antibody diluted in an antibody dilution buffer (1% BSA and 0.05% Tween20 in PBS) and incubated at 25 °C for 30 min. The plate was washed five times with washing solution, and Tetra Methyl Benzidine buffer (TMB PLUS2: Kementec, Taastrup, Denmark) was added. After a 30-min incubation, stop buffer (1.5N sulfuric acid) was added, and the absorbance of the sample (wavelength 450 nm) was measured.

Study population

In this study, we enrolled 52 cases of hospitalized patients with heart failure treated at Nagoya University Hospital between December 2019 and April 2021. The diagnosis of heart failure was established according to the European Society of Cardiology guidelines[6]. The conditions that led to hospitalization were acute decompensated heart failure due to progressive fluid retention, acute pulmonary edema, and cardiogenic shock and organ damage due to low cardiac output. Admissions were defined as those requiring diuretics, vasodilators, intravenous inotropes or antihypertensive medications, oxygen therapy, mechanical support, or surgical intervention. All patients underwent comprehensive clinical assessments, including detailed medical history, physical examination, and venous blood sample analysis. Clinical data collection and physical examinations were conducted by trained registry investigators using standardized clinical forms, while UCN2 blood samples were collected along with BNP samples immediately prior to discharge when heart failure symptoms had stabilized. Blood samples for laboratory parameters other than troponin were taken at the same time as UCN2 and BNP samples, and troponin blood samples were taken during hospitalization.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) or standard error of the mean. Continuous variables are presented as mean ± SD for normally distributed data or as median [interquartile range (IQR)] for non-normally distributed data. Categorical variables are presented as counts and proportions. The patients were divided into two groups according to the median UCN2 concentration, and the two groups were compared with respect to clinical, echocardiographic, and analytical parameters. Normality of continuous variables was

assessed using the Shapiro–Wilk test. Comparison between groups was performed using the chi-square test for categorical variables, independent samples t-test for normally-distributed continuous variables, and Mann–Whitney U-test when distribution was skewed. Univariate analysis was conducted using Spearman correlation coefficients, as the assumptions of normality and linearity were not met. Statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Software Inc. San Diego, CA, USA). "*n*" refers to the number of independent experiments per group. Statistical significance was defined as $p < 0.05$.

Data availability

All data are available in this manuscript and supplementary files.

Received: 16 December 2024; Accepted: 21 April 2025
Published online: 24 April 2025

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Acknowledgements

This study was supported by the Japan Society for the Promotion of Science KAKENHI.

Author contributions

K.M., K.K., K.F., and N.F. created the ELISA kit. K.I. and T.O. performed the statistical analyses. K.K., Y.Y., K.O., N.O., and T.M. provided advice throughout the study. R.M., T.K., H.H., S.K., T.Y., S.Y., and T.H. collected the clinical samples. M.T. was in charge of the research planning, statistical analysis, and manuscript writing. All authors reviewed the manuscript.

Declarations

Competing interests

K. Miyashita, K. Fukamachi, and N. Fukui are employees of Immuno-Biological Laboratories. The remaining authors declare no potential conflict of interest.

Ethics declarations

This study protocol complied with the Declaration of Helsinki and was approved by the Ethics Review Board of Nagoya University. Written informed consent was obtained from all participants.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-99509-4.

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