

High-Sensitivity Sandwich ELISA for Plasma NT-proUcn2: Plasma Concentrations and Relationship to Mortality in Heart Failure

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BACKGROUND: Urocortin 2 (Ucn2) has powerful hemodynamic, renal, and neurohormonal actions and likely participates in normal circulatory homeostasis and the compensatory response to heart failure (HF). A validated assay for endogenous circulating Ucn2 would facilitate investigations into Ucn2 physiology and elucidate its derangement and potential as a biomarker in heart disease.

METHOD: We developed a chemiluminescence-based sandwich ELISA to measure plasma N-terminal (NT)-proUcn2 in non-HF patients (control; n = 160) and HF patients with reduced (HFREF; n = 134) and preserved (HFPEF; n = 121) left ventricular ejection fraction (LVEF).

RESULTS: The ELISA had a limit of detection of 8.47 ng/L (1.52 pmol/L) and working range of 23.8–572 ng/L. Intra- and interassay CV and total error were 4.8, 16.2, and 17.7%, respectively. The median (interquartile range) plasma NT-proUcn2 concentration in controls was 112 (86–132) ng/L. HFREF, HFPEF, and all HF plasma concentrations were significantly increased [117 (98–141) ng/L, $P = 0.0007$; 119 (93–136) ng/L, $P = 0.0376$, and 119 (97–140) ng/L, $P = 0.001$] compared with controls but did not differ significantly between HFREF and HFPEF. NT-proUcn2 was modestly related to age ($r = 0.264$, $P = 0.001$) and cardiac troponin T ($r = 0.258$, $P = 0.001$) but not N-terminal pro-B-type natriuretic peptide, body mass index, LVEF, or estimated glomerular filtration rate. On multivariate analysis, plasma NT-proUcn2 was independently and inversely related to 2-year mortality in HF.

CONCLUSIONS: The validated ELISA measured human NT-proUcn2 in plasma and showed modest but significant increases in HF patients compared with controls. In HF, the unusual inverse relationship between plasma NT-proUcn2 and 2-year mortality portends potential prognostic value but requires further corroboration.

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The urocortins (Ucn1, Ucn2, and Ucn3), members of the corticotropin releasing factor (CRF)⁴ family, have diverse bioactivities and participate in the neuroendocrine responses to stress, appetite suppression, immunomodulation, energy and water homeostasis, and cardiovascular function (1–5). Their actions are mediated through the 7-transmembrane G-protein coupled receptors CRFR1 and CRFR2. Ucn1 binds to both receptors, whereas Ucn2 and Ucn3 are specific to CRFR2, widely expressed in the brain, heart, vasculature, and peripheral tissues (6–9). Binding of Ucn2 to its receptor triggers an intricate signaling cascade via protein kinase C, phosphatidylinositol-3-kinase/Akt and mitogen-activated protein kinase pathways to modulate cytokine expression, cell proliferation, and apoptotic responses (10–12). The presence of Ucn2 and CRFR2 in the heart and vasculature suggest a potentially important role for Ucn2 in the physiology and pathophysiology of the circulation in health and cardiovascular disease (13). Urocortins exert beneficial effects in experimental cardiovascular disease models and may have therapeutic potential (14–17).

Previously, we reported the generation and characterization of monoclonal antibodies targeting human

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⁴ Nonstandard abbreviations: CRF, corticotropin releasing factor; HF, heart failure; NT-proUcn2, N-terminal-proUrocortin 2; NT-proBNP, N-terminal pro-B-type natriuretic peptide; hs-cTnT, high-sensitivity cardiac troponin T; GAR-HRP, goat anti-rabbit IgG conjugated to horse radish peroxidase; HFREF, HF with reduced ejection fraction; LVEF, left ventricular ejection fraction; HFPEF, heart failure with preserved ejection fraction; mAb, monoclonal antibody; TrxUCN2pro, recombinant protein comprising NT-proUcn2 fused to C-terminus of thioredoxin; LC/Q-TOF, liquid chromatography quadrupole TOF; PBST, phosphate buffer containing 0.05% Tween 20, pH 7.4; RLU, relative luminescence units; LOD, limit of detection; LLOQ, lower limit of quantification; eGFR, estimated glomerular filtration rate; BMI, body mass index; AUC, area under the ROC curve.

Ucn2 prohormone and postulated the value of measuring endogenous proUcn2 as a surrogate measure of mature Ucn2 (18). The few reports to date on plasma Ucn2 in clinical cardiovascular disease offer implausibly discrepant values spread over a thousand-fold range for which little assay validation is available. Topal et al. (19) reported plasma Ucn2 concentrations in heart failure (HF) patients with systolic dysfunction in the range of 4.2–18.6 ng/L ($n = 56$), whereas Emeto et al. (20) reported a far higher median concentration of 2200 ng/L (interquartile range 1140–4550 ng/L; $n = 67$) in patients with abdominal aortic aneurysm. These authors used commercial ELISA assay kits from Cusabio Biotech and USCN Life Sciences, both assays ostensibly directed toward mature human Ucn2 (19, 20). In 2 previous studies, we used an in-house–developed 2-site ELISA applied to samples from volunteers and patients receiving infusions of exogenous Ucn2 and found plasma Ucn2 concentrations of 1280 and 5690 ng/L in healthy individuals and 1390 and 5360 ng/L in HF patients following infusion of 25 and 100 μg Ucn2 peptide, respectively (21, 22). However, endogenous circulating concentrations of Ucn2 in healthy individuals have not been evaluated.

Ucn2 processing in humans is unknown because the peptide gene products have not been successfully isolated from native tissues. Precursor and mature sequences are deduced by analogy to established processing sites of structurally related neuropeptides. Here we describe a sandwich ELISA directed against human N-terminal-proUrocortin 2 (NT-proUcn2) and report plasma concentrations in control study participants and HF patients together with demographic and clinical associates, comparisons with current benchmark biomarkers N-terminal pro-B-type natriuretic peptide (NT-proBNP) and high-sensitivity cardiac troponin T (hs-cTnT), and the relationship of plasma NT-proUcn2 to mortality in HF.

Materials and Methods

MATERIALS

Microplates (Black and White Isoplate-96/HB) were purchased from PerkinElmer. Super ChemiBlock was from Millipore, BSA from Sigma-Aldrich, polyclonal goat antirabbit IgG conjugated to horseradish peroxidase (GAR-HRP) from Dako, and Supersignal ELISA Femto Maximum Sensitivity Substrate and Pierce® goat anti-mouse IgG–coated 96-well microplates from Thermo Scientific. Control EDTA-anticoagulated human plasma (pooled from 10 healthy white donors age 30–80 years) that contained very low concentrations of endogenous Urocortin 2 was obtained from Bioreclamation.

STUDY POPULATION

Clinical samples were obtained from the previously described Singapore Heart Failure Outcomes and Pheno-

types Study (23, 24). EDTA-anticoagulated plasma samples were collected from June 2010 to May 2013 from 255 patients (age 31–91 years) with a confirmed clinical diagnosis of HF and from 160 volunteers (age 36–81 years) with no history of coronary artery disease or HF recruited to the Singapore Longitudinal Aging Study (23, 24). Among patients hospitalized for acute decompensated HF, blood was collected after patients were stabilized and judged fit for discharge. Within 0–2 days of blood sampling, all patients and controls underwent transthoracic Doppler echocardiography according to the American Society of Echocardiography guidelines. HF patients were categorized into HF with reduced ejection fraction [HFREF; left ventricular ejection fraction (LVEF) $\leq 40\%$; $n = 134$] and preserved ejection (HFPEF; LVEF $\geq 50\%$; $n = 121$) according to baseline LVEF determined using the biplane method of disks. Medical history, physical examination, and 12-lead electrocardiogram were documented for all participants (Table 1). Written informed consent was obtained from all participants. The study protocol was approved by the National Health Group Domain Specific Review Board (NHG DSRB Reference Code: 2010/00114) and Institutional Review Board of the National University of Singapore (NUS-IRB Reference Code: 04–140).

NT-proBNP AND hs-cTnT MEASUREMENTS

NT-proBNP and hs-cTnT were measured by electrochemiluminescence immunoassays on the ELECSYS Cobas e411 immunoanalyzer (Roche Diagnostics). The measurement ranges of NT-proBNP and hs-cTnT assays were 5–35 000 ng/L and 3–10 000 ng/L respectively.

PREPARATION OF TEST PROTEINS, CALIBRATOR, AND ANTIBODIES

The mouse monoclonal antibody (mAb 2D7) directed against human NT-proUcn2 described previously (18) was used as the capture antibody. The assay calibrator was a bacterially produced recombinant protein (TrxUCN2pro) comprising thioredoxin fused to an His₆ tag and NT-proUcn2 with a predicted mass of 23564 Da (see Fig. 1A in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol62/issue6>). Its purity by SDS-PAGE was $>95\%$ (see online Supplemental Fig. 1B) and its identity was confirmed by liquid chromatography quadrupole TOF (LC/Q-TOF) mass spectrometry, which established its mass as 23431 Da (see online Supplemental Fig. 1C), consistent with its amino acid sequence but with removal of the N-terminal methionine as expected. Purified TrxUCN2pro was diluted to obtain calibrators yielding equivalent NT-proUcn2 concentrations between 23.8–572 ng/L (approximately 4.3–102 pmol/L; NT-proUcn2 molecular mass of 5585 Da). Test proteins for evaluating assay cross-reactivity to bovine,

Table 1. Baseline characteristics of controls and HFREF and HFPEF patients.^a

| | Control (n = 160) | HFREF (n = 134) | HFPEF (n = 121) |
|----------------------------------|------------------------------|-------------------------------|------------------------------|
| Clinical characteristics | | | |
| Age, years | 58 (11) | 59 (12) ^{[**]b} | 70 (11) ^{**} |
| Male, % | 51 | 62 ^[*] | 42 |
| Race (Chinese/Malay/Indian) | 111, 28, 17 | 85, 30, 19 | 89, 24, 8 |
| BMI, kg/m ² | 25 (22-27) | 25 (22-29) ^[*] | 26 (24-30) ^{**} |
| Heart rate, beats/min | 67 (10 ^{**}) | 79 (15) ^[**] | 70 (13) ^{*} |
| Systolic blood pressure, mmHg | 130 (119-142) ^{*} | 120 (110-133) ^[**] | 130 (120-145) |
| Diastolic blood pressure, mmHg | 75 (68-82) ^{*} | 70 (61-80) ^[*] | 67 (60-77) ^{**} |
| NYHA ^c class, % | {**} | [Not significant] | {**} |
| (I) | NA | 26 | 22 |
| (II) | NA | 55 | 61 |
| (III) | NA | 17 | 16 |
| (IV) | NA | 2 | 2 |
| Coronary artery disease, % | 0 | 57 ^[**] | 36 |
| Myocardial infarction, % | 0 | 35 | 27 |
| Atrial fibrillation/flutter, % | 1 ^{**} | 14 ^[**] | 36 ^{**} |
| Hypertension, % | 29 ^{**} | 64 ^[**] | 89 ^{**} |
| Stroke previously, % | 1 ^{**} | 11 | 11 ^{**} |
| Peripheral vascular disease, % | 0 ^{**} | 11 ^[*] | 3 ^{**} |
| Chronic respiratory disease, % | 4 | 7 | 7 |
| Diabetes mellitus, % | 9 ^{**} | 58 | 58 ^{**} |
| Echocardiographic data | | | |
| LVEF, % | 63 (62-66) ^{**} | 26 (20-33) ^[**] | 60 (55-65) ^{**} |
| Laboratory investigations | | | |
| Creatinine, μmol/L | 69 (52-80) ^{**} | 104 (77-128) | 106 (79-138) ^{**} |
| eGFR, mL/min | 103 (85-110) ^{**} | 61 (47-86) | 55 (39-75) ^{**} |
| Medication | | | |
| ACE inhibitors, % | 3 ^{**} | 67 ^[**] | 45 ^{**} |
| Angiotensin receptor blockers, % | 4 ^{**} | 30 ^[*] | 44 ^{**} |
| Loop/thiazide diuretics, % | 4 ^{**} | 92 | 87 ^{**} |
| β blockers, % | 11 ^{**} | 96 ^[**] | 84 ^{**} |
| Statins, % | 17 ^{**} | 86 | 91 ^{**} |
| Digoxin, % | 1 ^{**} | 34 ^[**] | 14 ^{**} |
| Calcium channel blockers, % | 18 | 15 ^[**] | 54 ^{**} |
| Antidiabetics, % | 8 ^{**} | 53 | 52 ^{**} |

^a Data reported as % (categorical variables), mean (SD) (continuous variables of normal distribution), median (25th-75th percentile) (continuous variable of nonnormal distribution) as appropriate. Comparison of variables between HF groups based on Pearson chi-square test (categorical), independent *t*-test (parametric), and Mann-Whitney *U*-test (nonparametric).

^b *, significant at *P* < 0.05; **, significant at *P* < 0.001; {}, control vs HFREF; [], HFREF vs HFPEF; {}, control vs HFPEF.

^c NYHA, New York Heart Association Classification; ACE, angiotensin-converting enzyme.

mouse, and rat NT-proUcn2 were produced as thioredoxin fusions by cloning strategies described previously (18). His₆-SUMO-eXact tag fused to prepro-Urocortin 2, as described previously, was used as antigen for rabbit polyclonal antibody production (18).

TWO-SITE SANDWICH ELISA

Capture mAb 2D7 (6 μg/mL in 50 mmol/L carbonate-bicarbonate buffer, pH 9.6) was added to a Black and White Isoplate-96/HB microplate (100 μL/well) and incubated overnight at 4 °C. After 4 washes with phosphate

buffer containing 0.05% Tween 20, pH 7.4; 300 μL /well (PBST), blocking was performed overnight with 300 μL of 1.4% BSA in PBST at 4 °C. Coated plates were washed 4 times with PBST before use. Calibration curves were generated with 6 calibrators at 23.8, 47.7, 95.3, 191, 381, and 572 ng/L of NT-proUcn2. Calibrators were prepared in assay buffer (PBST containing 5% mouse serum and 100 mg/L Super ChemiBlock) and added to control plasma in a 1:1 ratio. Each plasma sample was prediluted 2-folds with assay buffer. Calibrators, quality controls, and test samples were added in duplicates to the 2D7-coated microplate (100 μL /well) and incubated for 2.5 h at room temperature with shaking at 550 rpm. After 4 PBST washes, 150 μL per well of rabbit polyclonal antibody diluted 1:1000 in PBST/1% mouse serum was added and incubated at room temperature for 2 h without shaking. The wells were washed 4 times with PBST, 150 μL per well of GAR-HRP (diluted 1:20000 in PBST/1% mouse serum) was added followed by incubation for 1 h at 37 °C. After 4 PBST washes, 100 μL per well of Femto substrate was added and chemiluminescence signals [in relative luminescence units (RLU)] were measured after 2 min on the Enspire Multimode Microplate Reader (PerkinElmer). Results, interpolated from a 5-parameter logistic model (5-PL), were applied to the calibrators using the microplate reader Enspire® software.

VALIDATION STUDIES

The limit of detection (LOD) was defined as the concentration value derived from the mean RLU of 18 zero standard replicates plus 2 SDs. The mean LOD was established from 10 independent assays. The lower limit of quantification (LLOQ) was determined as the lowest analyte concentration at which the intraassay %CV was within 20% over 24 independent assays.

Intraassay variation was assessed for 25 different plasma samples (concentrations ranging from 16.3 to 384 ng/L) in quadruplicates from 5 independent assays. Interassay variation of 5 different samples (mean concentrations 30.1, 42.9, 58.3, 120, and 149 ng/L) was obtained from 12 independent assays (2 replicates per sample) performed over a period of 2 months. Total error was assessed by combining the within-run and between-run variance components computed as:

$$\sigma^2_{\text{Total}} = \sigma^2_{\text{Within-run}} + \sigma^2_{\text{Between-run}} \quad (1)$$

To evaluate recovery, TrxUCN2pro was spiked into different plasma samples to increase the NT-proUcn2 concentrations by 18.9, 35.2, 69.4, 135, and 255 ng/L. The amount of endogenous NT-proUcn2 present in the sample before spiking was also determined. All samples were measured in quadruplicate in 4 independent assays. The percentage of recovery was computed as:

$$\% \text{ Recovery} = \left(\frac{(\text{Concentration of spiked sample} - \text{Concentration of unspiked sample})}{\text{Assigned spiked concentration}} \right) \times 100 \quad (2)$$

To evaluate dilution linearity, 4 samples were diluted 2-, 4-, 8-, and 16-fold to obtain NT-proUcn2 concentrations within the dynamic range of the assay. Mean concentrations from duplicate measurements were back-calculated from the fold-dilution and plotted against the dilution factor. A horizontal plot was expected for perfect dilution linearity.

Assay specificity was evaluated via cross-reactivity of mAb 2D7 to bovine, rat, and mouse NT-proUcn2 by surface plasmon resonance on the ProteOn XPR36 (BioRad Laboratories). Purified 2D7 was immobilized on a GLC chip as described previously (18) and tested against 100 nmol/L of thioredoxin fusions of human, bovine, rat, and mouse NT-proUcn2.

To identify the immunocaptured entity in the assay, 100 μL per well of 2D7 (1 mg/L in PBST) was added to a goat anti-mouse IgG-coated microplate and incubated for 60 min at room temperature. Following 3 PBST washes, 100 μL of plasma diluted 1:2 with PBST was added to each well and incubated for 2.5 h at room temperature with shaking at 550 rpm. Unbound material was removed by 3 PBST washes. The 2D7/analyte complex was eluted serially from 48 wells incubated with either pooled HF or control plasma using 2 separate 100- μL aliquots of 0.1 mol/L HCl. Ten microliters of each aliquot was injected into the LC/Q-TOF mass spectrometer for analysis (2 tests per sample) using water/0.1% formic acid and acetonitrile/0.1% formic acid as mobile phases. Consistent peaks that appeared in the mass spectra from both injections of the same sample were obtained. Treatment by strong acids was expected to deglycosylate the captured Urocortin 2 profragments to facilitate mass identification.

STATISTICAL ANALYSIS

Data are presented as mean (SD) or median (25th–75th percentiles) as appropriate. Associations between continuous variables were assessed using scatter plots and calculation of Pearson's correlation coefficient. Intergroup comparisons of continuous variables were by Student's *t*-test. χ^2 tests were applied to categorical variables. Analyses of univariate and multivariate associations of plasma NT-proUcn2 and assessment of candidate independent predictors of 2-year mortality in HF were performed by multiple logistic regression. Receiver-operator analyses were also used to assess the predictive performance and derive optimally predictive values of NT-proUcn2, NT-proBNP, and hs-cTnT. A 2-sided *P*-value <0.05% was accepted as statistically significant.

Table 2. Summary of NT-proUcn2 assay characteristics and performance parameters.

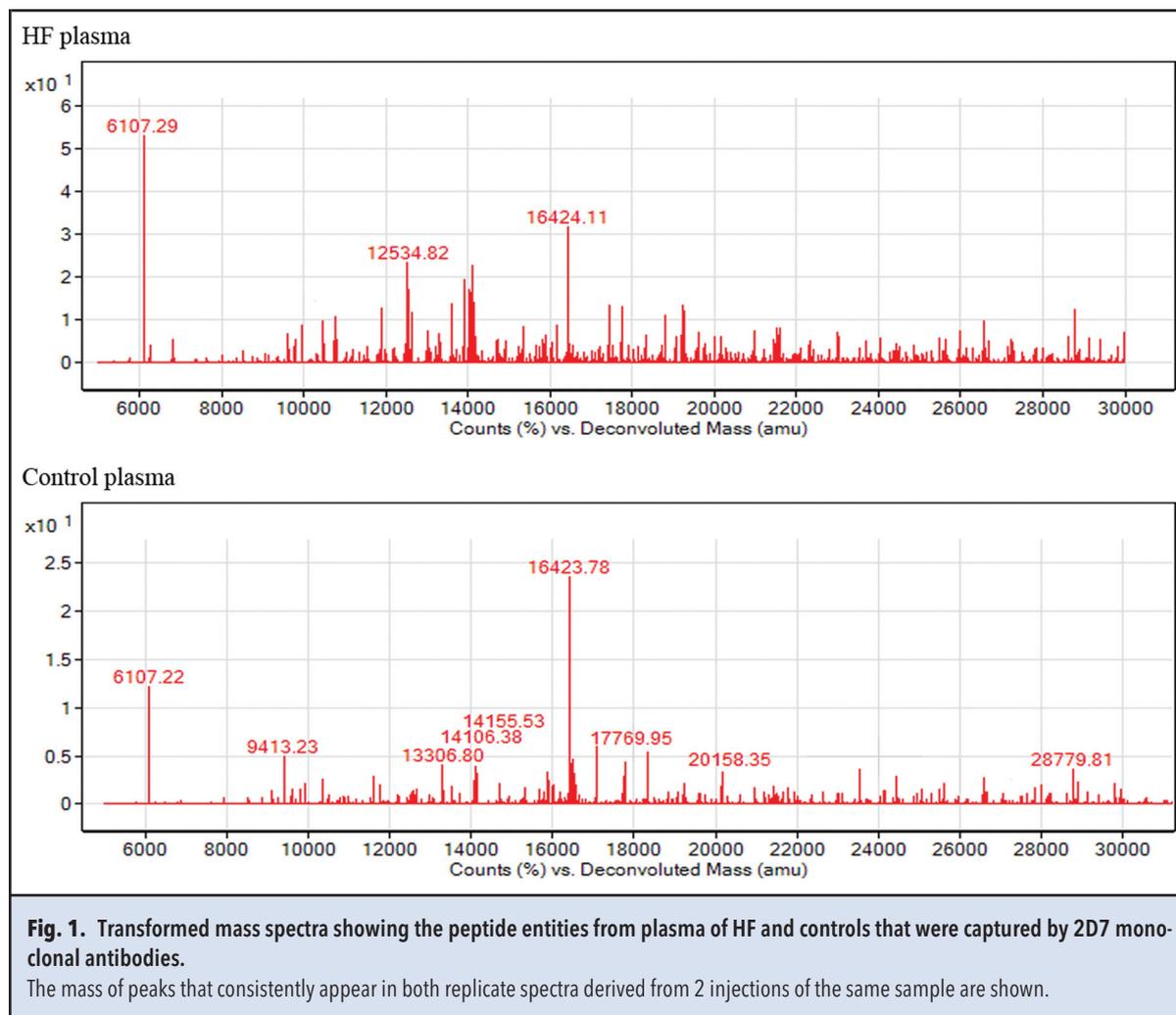
| Assay parameter | Assay characteristics | |
|---|---|-----------------------|
| Assay format | Sandwich ELISA/chemiluminescence | |
| Target | NT-proUcn2; human only | |
| Assay time | 5.5 h | |
| Assay range | 23.8–572 ng/L (4.3–102 pmol/L) | |
| Sample type | Plasma EDTA | |
| Amount of sample required | 50 µL plasma per well | |
| Total assay volume | 100 µL per well | |
| LOD (18 replicates, 10 independent assays) | Mean 8.47 ng/L (1.52 pmol/L); range 5.2–13.3 pg/mL; | |
| | Interassay %CV = 30.6 | |
| LLOQ (2 replicates, 24 independent assays) | 23.8 ng/L (4.3 pmol/L); | |
| | Intraassay %CV: range = 0.34%–16.8%; mean = 6.9% | |
| No detectable cross-reactivity to: | Human prepro-urocortin 1 and 3; bovine, mouse or rat NT-proUcn2 | |
| | Assay performance criteria | Observed results |
| Calibration curve R^2 | >0.99 | >0.99 to 1.0 |
| Signal-to-noise ratio | >8 | >8 |
| Whole plate uniformity (n = 6; raw luminescence signals) | %CV <10 | Mean %CV = 8.1 |
| | | Median %CV = 4.7 |
| | | Range %CV = 2.04–23.6 |
| Intraassay variation (within-run) (25 samples between 16.3–384 ng/L, 4 replicates per assay) | %CV <20 | Mean %CV = 4.8 |
| | | Median %CV = 3.9 |
| | | Range %CV = 0.5–20.7 |
| Interassay variation (between-run) (5 samples between 30.1–149 ng/L, 2 replicates per sample, 12 independent assays) | %CV <20% | Mean %CV = 16.2 |
| | | Median %CV = 16.1 |
| | | Range %CV = 13.8–19.4 |
| Total error (within- and between-run) (5 samples between 30.1–149 ng/L, 2 replicates per sample, 12 independent assays) | %CV <30 | Mean %CV = 17.7 |
| | | Median %CV = 17.8 |
| | | Range %CV = 14.7–20.7 |
| Spike and recovery (5 samples between 18.9–255 ng/L; 4 replicates per sample, 4 independent assays) | 80%–120% | 92.5%–117.7% |

Results

NT-proUcn2 ASSAY PERFORMANCE

NT-proUcn2 ELISA characteristics, assay acceptance criteria, and assay performance are summarized in Table 2. The assay working range of 23.8–572 ng/L was established on the basis of the precision profile for which the %CV for each calibrator did not exceed 20% in 24 inde-

pendent assays performed, with the dose–recovery plot providing further evidence of the tight concordance between the interpolated calibrator concentrations and that of the expected (see online Supplemental Fig. 2A–C). The mean LOD was 8.47 ng/L (approximately 1.52 pmol/L). Intraassay CVs ranged from 0.5% to 20.7% (mean 4.8%) for 25 different test samples between 16.3 and 384 ng/L of NT-proUcn2. Interassay CVs for con-



centrations between 30.1 and 149 ng/L were 13.8%–19.4% with a mean of 16.2%. Total error ranged between 14.7% and 20.7% with a mean of 17.7% (see online Supplemental Table 1). Recovery of spiked NT-proUcn2 was 92.5% to 118% within the range of 18.9–255 ng/L (see online Supplemental Table 2). The % recoveries obtained at 18.9 ng/L of spiked NT-proUcn2 ranged between 97% and 111% and were within $\pm 20\%$ of the assigned values, indicating that the lowest calibrator standard of 23.8 ng/L was within the quantifiable range. Acceptable linearity was obtained at 4-, 8-, and 16-fold dilutions for all tested samples (see online Supplemental Fig. 2D). However, the observed concentrations at 2-fold dilution were higher compared with those at higher dilutions, possibly due to matrix effects. NT-proUcn2 measurements were unaffected by up to 4 freeze–thaw cycles (see online Supplemental Fig. 2E). Microplate spatial uniformity tested at 2 concentrations that reflected low and high NT-proUcn2 plasma concentrations indicated that 1, possibly 2, out of 6 plates may

have well-to-well variability with CVs at or above 10% (see online Supplemental Fig. 3). This was consistent with the well-known row and/or column effects within microplates as well as systematic differences between them (25–27).

Surface plasmon resonance analysis showed that mAb 2D7 was specific to human NT-proUcn2 and did not cross-react with the bovine, mouse, or rat counterparts (see online Supplemental Fig. 4A). Two 2D7-captured protein entities (molecular masses of 6107 and 16423 Da) were common in both HF and control plasma (Fig. 1). Surprisingly, the 6107-Da product corresponded to a 57-residue (Gly₁₅–Arg₇₁) profragment (calculated mass, 6109.8 Da). The other product could not be attributed to any other fragment of the proUcn2 sequence.

NT-proUcn2 CONCENTRATIONS IN CONTROL AND HF PATIENTS

Table 1 lists and compares the demographic, clinical, echocardiographic, and biomarker data for the control, HFREF, and HFREF participants. Fig. 2 shows plasma

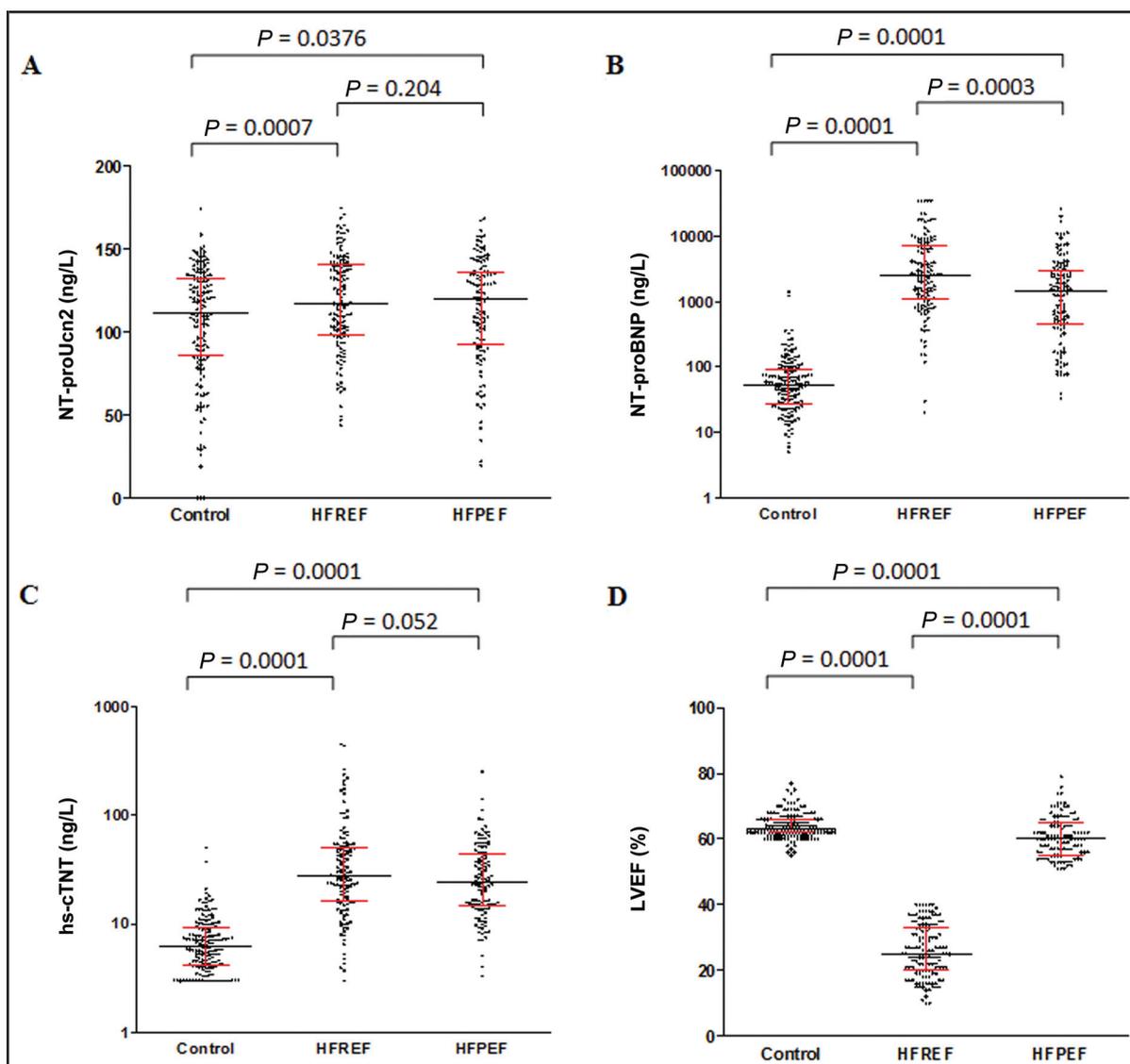


Fig. 2. Plasma (A) NT-proUcn2, (B) NT-proBNP and (C) hs-cTnT, and (D) LVEF (%) in HFREF (n = 134) and HFPEF (n = 121) compared with control participants (n = 160).

To aid visual distinction between groups by plasma NT-proUcn2 concentrations, 1 very high concentration data point for HFREF NT-proUcn2 at 324 ng/L was omitted from the Fig. (although included in analyses). The black, upper red, and lower red lines represent the median, 25th and 75th percentile, respectively.

concentrations of NT-proUcn2, NT-proBNP, and hs-cTnT in control, HFREF, and HFPEF. Median plasma NT-proUcn2 concentrations in HFREF of 117 (98–141) ng/L and HFPEF of 119 (93–136) ng/L were significantly higher than in controls [112 (86–132) ng/L; $P = 0.0007$ and $P = 0.0376$, respectively] although concentrations did not differ significantly between HFREF and HFPEF. As expected, NT-proBNP concentrations were markedly increased in HF with median values of 2550 (1104–7199) ng/L and 1464 (458–2956) ng/L in

HFREF and HFPEF, respectively, compared with 54 (28–493) ng/L in controls ($P = 0.0001$ for both comparisons). Concentrations in HFREF also differed significantly from those in HFPEF ($P = 0.0003$). Median plasma hs-cTnT concentrations were 6.1 (4.2–9.1) ng/L, 27.8 (16.3–50.1) ng/L, and 24.6 (14.8–44.4) ng/L in controls, HFREF and HFPEF respectively. Plasma hs-cTnT concentrations in both HFREF and HFPEF differed significantly from control ($P = 0.0001$ for both comparisons) whereas HFREF and HFPEF did not differ

significantly from one another ($P = 0.052$). Despite a trend toward increased plasma NT-proUcn2 with increased severity of NYHA functional class, concentrations did not significantly differ either between NYHA class I (median 111 ng/L) compared with classes II/III/IV combined (median 120 ng/L; $P = 0.866$) nor between classes I/II (117 ng/L) compared with classes III/IV (124 ng/L; $P = 0.860$). In contrast, median NT-proBNP was significantly lower in NYHA class I (840 ng/L) than in classes II/III/IV (2418 ng/L; $P < 0.001$). Similarly, hs-cTnT differed between class I and classes II/III/IV (medians 18.7 vs 30.2 ng/L; $P < 0.001$).

In controls ($n = 160$), plasma NT-proUcn2 was modestly associated with age ($r = 0.264$, $P = 0.001$) and hs-cTnT ($r = 0.258$, $P = 0.001$) but not NT-proBNP, estimated glomerular filtration rate (eGFR), body mass index (BMI), or LVEF. These findings differed from those for NT-proBNP and hs-cTnT, which both displayed correlations with all these variables, in accordance with previous reports (see online Supplemental Table 3). In further contrast to NT-proBNP and hs-cTnT, NT-proUcn2 did not differ according to sex or the presence of hypertension or diabetes within the control group. In HF, whether considering all cases combined ($n = 255$) or HFREF ($n = 134$) and HFPEF ($n = 121$) subgroups separately, plasma NT-proUcn2 concentrations were not significantly associated with age, sex, history of hypertension or diabetes, eGFR, BMI, LVEF, NT-proBNP, or hs-cTnT (see online Supplemental Table 4).

Multiple linear regression analyses on the entire study population for possible independent predictors of plasma NT-proUcn2 concentrations indicated an effect from study group (i.e., control or HF), which interacted with age ($P = 0.003$) when only these variables were considered. However, with addition of further candidate associates (including sex, hs-cTnT, LVEF, eGFR, BMI, and hypertension), no independent associates were apparent and the model explained very little of the interindividual variation in plasma NT-proUcn2 observed (adjusted $R^2 = 0.012$).

Thirty-five HF patients died over 2 years of follow-up. NT-proBNP [3524 (1827–1229) ng/L vs 1540 (645–3589); $P < 0.001$] and hs-cTnT concentrations [48.8 (27.6–66.9) vs 23.6 (14.6–43.8); $P < 0.001$] were significantly higher in decedents than in survivors. In contrast, plasma NT-proUcn2 was significantly lower in decedents than in survivors [105 (34) ng/L vs 119 (33) ng/L; $P = 0.029$]. Multivariate analyses for identification of independent predictors of 2-year mortality revealed NT-proUcn2 to be inversely related to 2-year mortality [risk ratio 0.99 (0.96–1.00); $P = 0.049$] independent of NT-proBNP (which exhibited the expected direct independent association with mortality), age, and LVEF. Each nanogram per liter increase in plasma NT-proUcn2 corresponded to a 1% fall in risk of death at 2 years. The

ROC-derived optimal plasma concentration of NT-proUcn2 for prediction of 2-year mortality was 103 ng/L with an associated area under the ROC (AUC) of 0.61 ($P < 0.001$). Mortality was significantly higher in those below compared with above this value of NT-proUcn2 ($P = 0.001$). In contrast, plasma concentrations above, rather than below, the ROC-derived optimally predictive values of both NT-proBNP (2919 ng/L; AUC 0.698) and hs-cTnT (37.2 ng/L; AUC 0.74) were associated with significantly higher mortality ($P < 0.001$ for both).

Discussion

Here we report a sandwich ELISA targeting the NT-proregion of human Urocortin 2 and its application in assessing NT-proUcn2 concentrations in health and HF. NT-proUcn2 concentrations ranged between nondetectable to a maximum of 324 ng/L, with the highest observed endogenous concentration readily measured well within the assay working range. Therefore, it is unlikely that samples with very high endogenous Ucn2 concentrations requiring >2-fold dilution would be encountered. The assay is specific to human NT-proUcn2 and does not cross-react with human prepro-Urocortin 1 and 3 (18) nor to bovine, mouse, and rat NT-proUcn2. The latter observation is concordant with multiple alignment of the animal peptide sequences against the human counterpart showing minimal conservation of the proregion (see online Supplemental Fig. 5).

Recent evidence from Ucn2 prohormone isolated from transduced CHO cells and melanoma cells point to signal peptide cleavage at Pro₂₄ without further processing of the prohormone (28). We found a peptide fragment corresponding to the profragment (Gly₁₅–Arg₇₁) in HF and control plasma, suggesting signal peptide processing after Leu₁₄ and processing of the Ucn2 precursor at Arg₇₁. These discrepancies may reflect different tissue origins of the isolated molecules in the 2 studies; the former observations perhaps arising from aberrant cleavage at Pro₂₄ by proline-specific proteases commonly observed in cancer cells (29) with subsequent loss of signal peptide and consequent failure of protein translocation across the endoplasmic reticulum for appropriate prohormone processing in the secretory pathway. Our data provides the natural processing sites for human Urocortin 2.

Plasma NT-proUcn2 in asymptomatic controls was significantly, albeit modestly, related to age but not to other common associates of the benchmark cardiovascular biomarkers (NT-proBNP and hs-cTnT) including renal function, LVEF, and BMI, or presence/absence of hypertension or diabetes. An association was found between plasma NT-proUcn2 and plasma hs-cTnT in these asymptomatic controls, raising a possible connection with healthy cardiac cell turnover or apoptosis. However, this finding requires corroboration and, overall, our cur-

rent data provide little guidance on the determinants of plasma NT-proUcn2.

Plasma NT-proUcn2 was significantly increased above the reference interval especially in HFREF but also in HFPEF, whereas the numerical difference between HFREF and HFPEF did not attain significance. The increases were modest and in health and disease NT-proUcn2 circulated over a relatively narrow concentration range compared with NT-proBNP. Unlike NT-proBNP and hs-cTnT, plasma NT-proUcn2 was inversely associated with 2-year mortality independent of NT-proBNP (which exhibits a directionally opposite relationship with mortality), age, and LVEF. The inverse relationship between NT-proUcn2 and mortality is an uncommon finding among cardiovascular biomarkers in HF. Its significance is uncertain. Ucn2 certainly has a multitude of protective effects upon the myocardium as demonstrated in numerous reports of preclinical and clinical data. Ucn2 exerts favorable effects on hemodynamics, renal function, and neurohormonal status in experimental ovine HF (30). Integrated renal, hemodynamic, and neurohormonal effects demonstrated in healthy volunteers and patients with chronic HF (21, 22) has further excited interest in the therapeutic potential of urocortins or analogs in human cardiovascular disease including HF and hypertension (31). Chan et al. infused Ucn2 in clinical HF and reported major effects upon cardiac output and arterial pressure (32). In view of this background, our results indicate that those patients with HF who are able to muster a more vigorous Ucn2 response receive some protection through that response. Corroboration of our finding (of an inverse relationship between plasma NT-proUcn2 and mortality in HF), its interpretation and elucidation of underlying mechanisms will require further work in other independent, clinical cohorts and in preclinical models with assessment of serial dynamic changes in expression and circulating concentrations of NT-proUcn2 in response to cardiac stress. It also warrants inspection of the cardiac response and any effect upon survival of manipulating NT-proUcn2 expression, secretion and achieved circulating concentra-

tions in NT-proUcn2 transgenic and knock-out models in the setting of experimental cardiac failure. Notably, the cardiac natriuretic peptides also exhibit an array of cardioprotective actions, and yet plasma concentrations are directly not inversely related to mortality in HF. This presumably reflects a compensatory response, proportional to the severity of cardiac dysfunction that has effectively been overwhelmed resulting in clinical HF.

In conclusion, we validated an immunoassay for plasma NT-proUcn2 and observed increased plasma concentrations in clinical HF along with an inverse relationship to 2-year mortality in HF. Although the modest increase in NT-proUcn2 concentrations in HF may limit its utility as a diagnostic marker, the unusual inverse relationship to 2-year mortality portends its potential prognostic value and requires further careful investigations.

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References

1. Bale TL, Vale WW. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* 2004;44:525-57.
2. Fekete EM, Zorilla EP. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. *Front Neuroendocrinol* 2007;28:1-27.
3. Kuperman Y, Chen A. Urocortins: emerging metabolic and energy homeostasis perspectives. *Trends Endocrinol Metab* 2008;19:122-9.
4. Stengel A, Taché Y. CRF and urocortin peptides as modulators of energy balance and feeding behaviours during stress. *Front Neurosci* 2014;8:1-10.
5. Zorilla EP, Logrip ML, Koop GF. Corticotropin releasing factor: A key role in the neurobiology of addiction. *Front Neuroendocrinol* 2014;35:234-44.
6. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 1995;378:287-92.
7. Hsu SY, Hsueh AJ. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat Med* 2001;7:605-11.
8. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A* 2001;98:7570-5.
9. Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, et al. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A* 2001;98:2843-8.
10. Emoto TI, Moxon JV, Rush C, Woodward L, Gollidge J. Relevance of urocortins to cardiovascular disease. *J Mol Cell Cardiol* 2011;51:299-307.
11. Li J, Qi D, Cheng H, Hu X, Miller EJ, Wu X, et al. Urocortin 2 autocrine/paracrine and pharmacologic effects to activate AMP-activated protein kinase in the heart. *Proc Natl Acad Sci U S A* 2013;110:16133-8.
12. Onorati F, Chen-Scarabelli C, Knight R, Stephanou A,

- Mohanti B, Santini F, et al. Targeting urocortin signaling pathways to cardioprotection: is it time to move from bench to bedside? *Cardiovasc Drugs Ther* 2013; 27:451–63.
13. Richards AM, Rademaker MT. Urocortins. In Kastin AJ, editor. *Handbook of biologically active peptides*, 2nd ed. Amsterdam: Elsevier Inc; 2013. p 1428–36.
 14. Davidson SM, Rybka AE, Townsend PA. The cardioprotective effects of urocortin and the corticotropin releasing hormone (CRH) family. *Biochem Pharmacol* 2009; 77:141–50.
 15. Venkatasubramanian S, Newby DE, Lang NN. Urocortins in heart failure. *Biochem Pharmacol* 2010;80:289–96.
 16. Bühler K, Plaisance I, Dieterle T, Brink M. The human urocortin 2 gene is regulated by hypoxia: identification of a hypoxia-responsive element in the 3' flanking region. *Biochem J* 2009;424:119–27.
 17. Hashimoto K, Nishiyama M, Tanaka Y, Noguchi T, Asaba K, Hossein PN, et al. Urocortins and corticotropin releasing factor type 2 receptors in the hypothalamus and the cardiovascular system. *Peptides* 2004;25:1711–21.
 18. Liew OW, Ang CX, Peh YP, Chong PC, Ng YX, Hwang LA, et al. A His₆-SUMO-eXact tag for producing prepro-Urocortin 2 in *Escherichia coli* for raising monoclonal antibodies. *J Immunol Methods* 2014;403:37–51.
 19. Topal E, Yagmur J, Otlu B, Atas H, Cansel M, Acikgoz N, Ermiş N. Relationship of urocortin-2 with systolic and diastolic functions and coronary artery disease: an observational study. *Anadolu Kardiyol Derg* 2012;12: 115–20.
 20. Emoto TI, Moxon JV, Biros E, Rush CM, Clancy P, Woodward L, et al. Urocortin 2 is associated with abdominal aortic aneurysm and mediates anti-proliferative effects on vascular smooth muscle cells via corticotrophin releasing factor receptor 2. *Clin Sci* 2014;126:517–27.
 21. Davis ME, Pemberton CJ, Yandle TG, Fisher SF, Lainchbury JG, Frampton CM, et al. Urocortin 2 infusion in healthy humans: hemodynamics, neurohormonal and renal responses. *J Am Coll Cardiol* 2007;49:461–71.
 22. Davis ME, Pemberton CJ, Yandle TG, Fisher SF, Lainchbury JG, Frampton CM, et al. Urocortin 2 infusion in human heart failure. *Eur Heart J* 2007;28:2589–97.
 23. Santhanakrishnan R, Ng TP, Cameron VA, Gamble GD, Ling LH, Sim D, et al. The Singapore Heart Failure Outcomes and Phenotypes (SHOP) study and Prospective Evaluation of Outcome in Patients with Heart Failure with Preserved Left Ventricular Ejection Fraction (PEOPLE) study: rationale and design. *J Card Failure* 2013;19:156–62.
 24. Santhanakrishnan R, Chong JP, Ng TP, Ling LH, Sim D, Leong KT, et al. Growth differentiation factor 15, ST2, high-sensitivity troponin T, and N-terminal pro brain natriuretic peptide in heart failure with preserved vs. reduced ejection fraction. *Eur J Heart Failure* 2012;14: 1338–47.
 25. Bauske EM, Hewings AD, Kolb FL, Carmer SG. Variability in enzyme-linked immunosorbent assays and control of experimental error by use of experimental designs. *Plant Dis* 1994;78:1206–10.
 26. Williams JD, Birch JB, Walfish SA. Statistical method to account for plate-to-plate variability in multiple plate bioassays. *BioPharm Int* 2003;May;44–54.
 27. Liang Y, Woodle SA, Shibeko AM, Lee TK, Ovanesov MV. Correction of microplate location effects improves performance of the thrombin generation test. *Thromb J* 2013;11:12.
 28. Vaughan JM, Donaldson CJ, Fisher WH, Perrin MH, Rivier JE, Sawchenko PE, Vale WW. Posttranslational processing of human and mouse Urocortin 2: Characterization and bioactivity of gene products. *Endocrinology* 2013;154:1553–64.
 29. Busek P, Malik R, Sedo A. Dipeptidyl peptidase IV activity and/or structure homologues (DASH) and their substrates in cancer. *Int J Biochem Cell Biol* 2004;36:408–21.
 30. Rademaker MT, Cameron VA, Charles CJ, Richards AM. Integrated hemodynamic, hormonal, and renal actions of urocortin 2 in normal and paced sheep: beneficial effects in heart failure. *Circulation* 2005;112:3624–32.
 31. Dieterle T, Meili-Butz S, Bühler K, Morandi C, John D, Buser PT, et al. Immediate and sustained blood pressure lowering by Urocortin 2: a novel approach to anti-hypertensive therapy? *Hypertension* 2009;53:739–44.
 32. Chan WYW, Frampton CM, Crozier IG, Troughton RW, Richards AM. Urocortin-2 infusion in acute decompensated heart failure: findings from the UNICORN study (urocortin-2 in the treatment of acute heart failure as an adjunct over conventional therapy). *JACC Heart Fail* 2013;1:433–41.