Decompensated Heart Failure is Associated with Reduced Corin Levels and Decreased Cleavage of Pro-Atrial Natriuretic Peptide

Running Title: Ibebuogu et al: Corin-ANP in Acute Heart Failure

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Abstract

Background—By promoting salt and water excretion, the corin and the atrial natriuretic peptide (ANP) system should help to maintain fluid balance in heart failure. Yet, the development of fluid retention despite high levels of ANP-related peptides, suggests that this compensatory system is limited.

Methods and Results—Levels of circulating corin (the pro-ANP converting enzyme) and pro-ANP were measured in hospitalized patients with heart failure, using novel immunoassays. Patients (n = 14) had severe heart failure (NYHA class III-IV) with a median ejection fraction of 18 % and median BNP levels of 1940 pg/ml. In heart failure, median plasma corin levels were 7.6-fold lower than measured in plasma from 16 normal controls (180 vs. 1368 pg/ml, p<0.01). In contrast, in heart failure patients, levels of plasma N-terminal ANP peptides (N-ANP and pro-ANP) levels were markedly elevated (42.0 vs. 7.5 ng/ml, p<0.01). Levels of uncleaved pro-ANP, measured by novel immunoassays, were significantly higher in heart failure patients (p < 0.01) suggesting that corin cleavage of pro-ANP was impaired. Median plasma levels of cGMP were elevated in heart failure patients (150.0 vs. 7.6 pmol/ml, p<0.01) and plasma cGMP levels positively correlated with the fractional amount of cleaved pro-ANP (r₅ = 0.59, p < 0.03) but not with levels of uncleaved pro-ANP, implying that the cellular response to ANP remained intact.

Conclusions—Taken together these data suggest that there may be patients for whom low corin levels and impaired pro-ANP cleavage contribute to acute decompensation.

Key Words: atrial natriuretic peptide, natriuretic peptides, heart failure
Heart failure is a major public health problem that affects more than 4.5 million Americans; about 550,000 new cases are diagnosed each year\textsuperscript{1,2}. Despite treatment advances, heart failure is associated with high morbidity and, half of heart failure patients die within 5 years\textsuperscript{3}. For unknown reasons, individuals with the same degree of impaired heart function can show marked differences in the development of heart failure symptoms\textsuperscript{4,5}, suggesting that factors other than cardiac function contribute to this condition.

Corin is a serine protease in the heart\textsuperscript{6} that is primarily responsible for cleavage-activation of pro-atrial natriuretic peptide (pro-ANP) to ANP\textsuperscript{7}. Although corin expression has not been quantified in humans with heart failure, a previous study reported that cardiac tissue levels of corin were not altered in an autopsy specimen from a patient with total anomalous pulmonary venous return\textsuperscript{8}. Corin transcripts and protein are abundantly expressed in cardiomyocytes of both the atrium and ventricle, often in cells that also contain pro-ANP\textsuperscript{6,8,9}. Corin gene expression is increased in the hypertrophied regions of the ventricle of the heart after coronary ligation in rats\textsuperscript{10} but corin is decreased in the atrium after the development of experimental heart failure in rats\textsuperscript{11}. This suggests that the activity of corin and, the subsequent cleavage of pro-ANP, may be altered in humans who develop heart failure and that corin may be involved in the pathogenesis of heart failure.

In individuals with diminished heart function there is increased production of pro-ANP\textsuperscript{12-16}. Pro-ANP is a pro-hormone that is secreted from heart and cleaved to an N-terminal fragment (N-ANP) and a C-terminal, biologically active peptide (ANP) by the heart-specific serine protease corin (Figure 1)\textsuperscript{7,17}. ANP, but not pro-ANP, binds to the natriuretic peptide-A receptor (NPR-A) to initiate signaling through 3'-5'-cyclic
guanosine monophosphate (cGMP)\textsuperscript{18, 19}. Through this mechanism ANP triggers sodium excretion (natriuresis), causes vasodilation, interferes with the renin-angiotensin system, inhibits mitogenesis, and mediates other effects\textsuperscript{14, 20, 21}. These effects of the ANP can be beneficial in patients with impaired heart function and heart failure\textsuperscript{22} and there is evidence that infusions of ANP ameliorate heart failure\textsuperscript{23, 24}.

It is an apparent paradox that the highest levels of ANP-related peptides (pro-ANP, N-ANP and ANP) occur in patients with severe heart failure who have the worse prognosis\textsuperscript{25-28}. Why high levels of ANP-related peptides fail to avert heart failure is poorly understood. We hypothesized that defects in the corin-ANP system may occur in heart failure patients that lead to impaired cleavage of pro-ANP to the active peptide ANP. Unfortunately, current immunoassays can’t separately measure pro-ANP, N-ANP or ANP peptides, because they use antibodies directed to the N-terminal portion or C-terminal portion of the molecule. For example, immunoassays directed against N-terminal sequences in pro-ANP detect both pro-ANP and N-ANP; immunoassays directed against C-terminal sequences in ANP detect both pro-ANP and ANP. To examine if the corin-ANP system was altered in humans with heart failure, we developed novel assays to measure plasma levels of pro-ANP, N-ANP and corin.

\textbf{Methods}

\textbf{Study Design}

The study protocol and consent form were approved by IRB of the Medical College of Georgia, Augusta, Georgia, USA. Plasma samples were obtained from healthy normal blood donors (16 total) and from 14 patients admitted to the hospital with a diagnosis of
acute decompensated heart failure due to impaired heart systolic function (ejection fraction < 35%). Heart failure patients provided baseline demographics and medical history such as age, history of hypertension, hyperlipidemia, smoking, and family history of premature coronary heart disease. Heart failure patients were excluded who had conditions that might also affect the natriuretic system such as chronic kidney disease (estimated glomerular filtration rate <60ml/min/1.73m²), pulmonary hypertension (pulmonary artery pressure >55mmHg by transthoracic echocardiography), myocardial infarction within the last 6 weeks, critical valvular heart disease, metastatic or terminal cancer. Demographic data on controls were obtained from the Medical College of Georgia Blood Bank.

**Blood Sample Collection**

Venous blood samples were collected from consenting subjects on admission using standard EDTA-aprotinin tubes and immediately stored on ice. These inhibitors block enzymes in the blood known to affect the degradation of pro-ANP²⁹. The blood samples were centrifuged at 3000xg for 20 min at 4°C, aliquoted, and stored at -80°C until analysis.

**Heart failure Biomarkers Measurement**

Assays were performed by personnel unaware of the patient’s identity and outcome. BNP-related peptides were measured by the standard clinical laboratory method. Elevated BNP levels were defined as those values above the 95th percentile of normal³⁰.
The cGMP was measured with an enzyme immunoassay (R&D Systems, Inc., Minneapolis, MN).

Plasma N-terminal-related ANP peptides (pro-ANP + N-ANP) were measured using an enzyme immunoassay directed against an N-terminal amino acid sequence (26-55 of pre-pro ANP, or 1-30 of pro-ANP, EK-005-19, Phoenix Pharm., Inc.). This ELISA measures pro-ANP and N-ANP but has no reactivity with C-ANP-related peptides such as ANP. In order to separately measure pro-ANP and N-ANP we altered the assay process to immunodeplete all C-ANP-related peptides (pro-ANP and C-ANP but not N-ANP) from samples using an antibody directed against C-ANP (G-005-06, ANP1-28, Phoenix Pharm, Inc.). This process then allowed direct measurement of the N-ANP remaining in the plasma. Plasma samples were diluted with 0.05% Tween 20 in PBS and applied into a 96-well, U-bottom flexible microplate (Becton Dickinson Labware, Franklin Lakes, NJ) pre-coated with either purified rabbit anti-human C-ANP (1-28) IgG (5 μg/ml PBS, Phoenix Pharm., Inc) or with control rabbit IgG (5 μg/ml PBS) sealed and incubated for 1 h at room temperature. Subsequently we measured the N-ANP in the plasma depleted of C-ANP-related peptides or, both N-ANP and pro-ANP in control-depleted plasma samples (after pretreatment with control IgG) using the N-terminal enzyme immunoassay (EK-005-19, Phoenix Pharm., Inc.). In our assays, N-ANP (26-55) or purified recombinant pro-ANP (see below) was used as a control. Pro-ANP values in the plasma samples were determined by the difference between the measured N-ANP and the total N-ANP-related peptides (pro-ANP and N-ANP). To confirm the complete depletion of C-ANP related peptides (pro-ANP and C-ANP) in these assays we used an enzyme immunoassay directed against an C-terminal amino acid sequence (ANP 1-28).
contained in C-ANP (EK-005-06, Phoenix Pharm., Inc.) and recombinant pro-ANP proteins. Under these assay conditions the specific anti-C-ANP IgG, but not the control rabbit IgG, successfully depleted recombinant proteins at concentrations that exceed the highest values measured in all our plasma samples. When the ANP-related peptides were measured using this immunodepletion method the sensitivity of these assays increased.

Immunoblots were performed to identify pro-ANP in plasma. Plasma (1 ml) was incubated with immobilized antibody directed against full-length pro-ANP antibody (FL-153, Santa Cruz Biotechnology) for 2-4 hours at 4 deg. After washing, bound proteins were eluted with sample buffer (100º C) and subjected to SDS-PAGE under reducing conditions. After electrophoretic transfer, blots were probed with antibodies directed to sequences in the N-terminus (prepro-atrial natriuretic peptide (26-55), Bachem Americas) and the C-terminus of pro-ANP (ANP alpha (1-28), Phoenix Pharm.). Blots were probed with IRDye-680 goat-anti-rabbit secondary antibodies and exposed on Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences).

**Enzyme Immunoassay for human corin in plasma**

A sandwich ELISA assay was developed to measure human corin in plasma samples using DuoSet ELISA Development kit (R&D Systems). Plasma samples were diluted 2-3 fold with ELISA assay buffer, 0.05% Tween 20 in PBS, pH 7.2-7.4. To precipitate ballast proteins and amplify corin binding to the capture antibody, diluted plasma samples were applied for 40 min. to a 96-well, U-bottom flexible microplate (Becton Dickinson Labware, Franklin Lakes, NJ) pre-coated with non-specific rabbit IgG (5 µg/ml PBS) purified by protein A-Sepharose (Pierce, Rockford, IL). For the ELISA assay wells of a
flat-bottom microplate (R&D Systems) were pre-coated with the capture anti-human corin rat monoclonal antibody (4 μg/ml PBS) produced against the recombinant extracellular domain of corin 67-1042 amino acids (R&D Systems). The plate was sealed and incubated over night. Non-specific binding sites were block with 1% BSA in PBS for 1 h. Recombinant human corin protein was used as a standard for this assay. All assay steps were performed at room temperature and with orbital shaking. Plasma samples after pretreatment with control rabbit IgG or standards were applied to the plate pre-coated with capture antibody and incubated 2 h. After multiple washing with 0.05% Tween 20 in PBS, pH 7.2-7.4, captured corin was detected with biotinylated polyclonal goat anti-human corin antibody (raised against recombinant extracellular domain of corin spanning amino acids 67-1042 (R&D Systems). After incubation for 2 h followed by 4 washes, the wells were incubated with streptavidin-horseradish peroxidase for 30 min protected from light. After repeat washing, horse-radish peroxidase substrate solution (1:1 mixture of H₂O₂ and tetramethylbenzidine, R&D Systems) was added to the plate, and the reaction was stopped after 30 min by 2N HCl. Optical density was measured immediately at 450 nm, using a microplate reader (Bio-Tek Instruments Inc.). The assay sensitivity for corin was 30-50 pg/ml.

**Recombinant pro-ANP protein**

Pro-ANP cDNA was amplified from a Human Adult 8 Tissue GenePools cDNA library (NT Omics, Inc San Mateo, CA) by PCR. The pro-ANP cDNA was sequenced for confirmation, cloned into the pcDNA 3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) using the pcDNA3.1/V5-His-TOPO TA Expression Kit (Invitrogen) to yield the
plasmid, pcDNA-pro-ANP. Recombinant pro-ANP was expressed in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) using Lipofectin (Invitrogen) according to the manufacturer’s protocol. The pro-ANP in the conditioned medium (serum-free Opti-MEM, Invitrogen) was measured with C-ANP enzyme immunoassay (Phoenix Pharm., Inc.).

**Statistical Analysis**

Unless otherwise indicated, continuous data are presented in tabular form or in box plots indicating the medians with upper and lower quartile values. Data were analyzed by non-parametric methods (Mann Whitney tests and Spearman’s rank correlation- $r_s$). A two-tailed $p$-value < 0.05 was considered statistically significant.

**Results**

For the heart failure patients, the mean BNP level on hospital admission was 1940 pg/ml (Table 1) which is markedly higher than the upper limit of normal in our hospital (<100 pg/ml). The mean age of heart failure patients was 49 years and 57% were males. Half of the heart failure patients had ischemic cardiomyopathy and half had dilated cardiomyopathy. Most (93%) had severe heart failure symptoms (NYHA class III and IV). The median ejection fraction of 18% was significantly reduced.

In the initial immunoassay, corin was undetectable in the plasma from several heart failure patients, although it was readily measured in normal plasma samples. To increase the sensitivity of the test, we reconfigured the immunoassay to remove ballast proteins$^{32}$. Subsequently corin was measurable in all subjects. The median plasma corin
level was 7.6-fold lower in heart failure patients than normal controls (180 pg/ml vs. 1368 pg/ml, p<0.01; Figure 2). In immunoassays using our previously reported anti-corin monoclonal antibodies we also found that corin levels were markedly lower in patients with heart failure (data not shown). There were no significant differences in median corin levels between men and women (286 vs. 99 pg/ml, p=0.35).

Lower corin levels may signify a reduced capacity to cleave pro-ANP in patients with heart failure. An immunoassay, which detects the N-terminal ANP peptides N-ANP and pro-ANP, showed that these median peptide levels were significantly higher in patients with heart failure than in the plasma from normal individuals (42.0 vs. 7.5 ng/ml, p<0.01, Figure 3A). To assess the cleavage of pro-ANP, we developed an assay that allowed us to measure separately the levels of pro-ANP and N-ANP, a biologically stable cleavage product. Plasma samples were treated with a control, non-specific antibody or with an antibody directed against C-ANP related peptides to immunodeplete or remove ANP and pro-ANP. The C-ANP antibody completely removed ~1.4 nM of C-ANP related peptides from plasma (which exceeded the pro-ANP in our samples—see below), but the control antibody did not (Figure 3B). Pre-treatment with the C-antibody was specific in that it did not affect levels of N-ANP peptides measured by comparison to pre-treatment with the control antibody (Figure 3C). The remaining N-ANP peptide was then measured in the plasma immunodepleted of C-ANP-related peptides. In the heart failure patients the amount of cleaved pro-ANP (N-ANP) rose as the total levels of pro-ANP and N-ANP increased (r_s=0.58, p<0.03). In normal plasma, essentially all pro-ANP was cleaved to N-ANP and the circulating pro-ANP was not significantly different from 0 ng/ml (p=0.18). However, in the patients with heart failure, the levels of uncleaved pro-
ANP were significantly higher than in normal plasma (Figure 3D, \( p<0.01 \)) indicating that corin cleavage of pro-ANP was impaired in these patients. Pro-ANP could be detected in heart failure plasma but not normal plasma by immunoblotting (Figure 3E).

After cleavage of pro-ANP, ANP acts on cells through the natriuretic peptide receptor A to increase cGMP levels and execute its biological effects (Figure 1)\(^\text{18}\). Plasma levels of cGMP were much higher (20-fold) in patients with heart failure than in normal individuals (150 vs. 7.6 pmol/ml, \( p<0.01 \), Figure 4). Still, in these heart failure patients there was no significant correlation between the levels of cGMP and levels of N-terminal ANP-related peptides as measured by a conventional immunoassay (\( r_s = -0.47, p =0.09 \)). In addition there was no significant relationship between levels of cGMP and levels of pro-ANP (\( r_s = -0.42, p =0.14 \)). However, there was a significant positive correlation between the fractional amount of cleaved pro-ANP and cGMP levels (\( r_s = 0.59, p <0.03 \)).

**Discussion**

ANP is an important regulator of physiologic volume in heart failure through its effects on salt and water excretion\(^\text{33}\). The observation that patients develop worsening salt and water overload despite very high levels of ANP-related peptides has been perplexing. Indeed, the severity of heart failure is linked to levels of ANP-related peptides\(^\text{34-36}\).

While patients with severe heart failure develop high levels of ANP-related peptides and high levels of cGMP, our study and others have shown that there is no correlation between cGMP levels and total ANP-related peptides\(^\text{5,37}\). We postulated that one explanation for the loss of the relationship between levels of ANP-related peptides and
cGMP could be that corin levels and pro-ANP cleavage were reduced in severe heart failure. Consistent with this notion, in normal plasma corin was readily detectable (Figure 2) and pro-ANP was absent, because it was fully cleaved to N-ANP (Figure 3D). By comparison, in plasma from heart failure patients, corin was markedly decreased (Figure 2) and pro-ANP was readily detected because it was not all cleaved to N-ANP (Figure 3D). Still, in the heart failure patients, cGMP levels were positively correlated with the levels of cleaved pro-ANP, which suggests that at the cellular level, patients retain some degree of responsiveness to biologically active forms of ANP. Supporting this notion, recent studies in patients with severe heart failure have shown that infusions of ANP (carperitide) increase cGMP levels, which indicates that these patients retain responsiveness to biologically active forms of ANP at the cellular and receptor level.38

In addition to decreased corin levels and decreased pro-ANP cleavage, other defects not examined in this study, could occur in the natriuretic peptide system that may contribute to heart failure such as alterations in NPR clearance and signaling, the bioavailability of natriuretic peptides and, the degradation of natriuretic peptides.39, 40

While this paper was in review, a retrospective study by Rame et al. found that a polymorphism in corin I555 (P568) was associated with lower BNP and pro-BNP levels as well as worse outcomes in the A-HeFT study.41 It has been suggested that in some patients with severe heart failure, the responsiveness of the kidney to increasing levels of ANP-related peptides is diminished, although vasodilatory effects persist.42, 43 Renal hyporesponsiveness to ANP has been attributed to many potential factors including the counter-regulatory effects of the renin-angiotensin-aldosterone-sympathetic system.
There are limitations to the conclusions that can be drawn from this study. Since our study focused on acute heart failure by comparison to normal controls (blood donors), we are unable to determine whether changes in circulating corin levels and altered natriuretic processing are also seen in individuals with chronic heart failure, impaired systolic function without heart failure, or other cardiomyopathies or conditions. The relatively small sample in this study may have reduced our statistical power to detect correlations between levels of corin expression and age, sex or other variables. Due to limited information on the controls we are unable to determine whether other factors besides heart failure account for the differences we observed. In the absence of myocardial expression studies, we are unable to determine whether reduced corin levels reflect diminished cardiac production, cleavage, etc.

These studies demonstrate a plausible link between levels of uncleaved pro-ANP and low circulating corin in these patients with heart failure. Corin is a transmembrane protease originally identified in cardiomyocytes by homology to serine proteases and LDL-like molecules. Although corin can cleave pro-ANP and pro-BNP in vitro, there is only strong evidence that corin cleaves pro-ANP in vivo. In contrast, pro-BNP appears to be cleaved in secretory granules in the heart and the relationship between corin cleavage of pro-BNP and heart failure is unknown. Mice lacking corin have mild hypertension and lack detectable ANP. Polymorphisms in the corin gene have been associated with increased risk of hypertrophy in the setting of systolic hypertension. These polymorphisms are associated with decreased activation of corin (zymogen) and thereby decreased corin activity. Although there are no reports of circulating corin levels in humans, other transmembrane and matrix proteins such as P-selectin, matrix metalloproteases (1, 2, 9) and their endogenous tissue inhibitors circulate...
in the blood and have pathophysiologic significance in cardiovascular disease.\textsuperscript{53-59} Many of these molecules retain biological activity. Although we were unable to assess the proteolytic activity of corin in our samples (due to the addition of inhibitors to prevent proteolysis of pro-ANP and corin), it has been shown that soluble forms of recombinant corin cleave recombinant pro-ANP molecules in human plasma.\textsuperscript{60} Since the heart is the primary site for the production of corin, decreased plasma corin levels may reflect myocardial disease, but additional studies will be required to determine the linkage between myocardial corin expression, plasma corin levels and corin activity. The reduced plasma levels of corin may be due to diminished cardiac expression of this protein, enhanced protein cleavage or, production of splice variants that lack transmembrane domains.

This small study is the first to evaluate the corin-ANP system in humans and, larger studies, in more diverse populations, will be necessary to generalize our findings.\textsuperscript{61} Still, the ability to accurately measure both the cleaved and uncleaved forms of pro-ANP should provide further insights into the physiologic and pathologic regulation of the corin-natriuretic system. These assays may identify patients for whom acute decompensated heart failure is the outcome of impaired pro-ANP cleavage. They may also provide a means for phenotyping individuals for personalized treatment with ANP or other therapies. In addition, levels of circulating corin may have additional diagnostic and prognostic value as a surrogate marker in individuals with suspected heart disease.
Acknowledgments

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Disclosures

MCG has recently applied for patents related to this research. Otherwise none.
References


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Table. Characteristics of Heart Failure Patients and Controls

<table>
<thead>
<tr>
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<th>Heart Failure Patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>Median*</td>
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<tr>
<td></td>
<td>Range</td>
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<tr>
<td><strong>Gender</strong></td>
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<tr>
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<td><strong>Etiology</strong></td>
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</tr>
<tr>
<td></td>
<td>Dilated Cardiomyopathy</td>
<td>50%</td>
</tr>
<tr>
<td><strong>Admission BNP (pg/ml)</strong>*</td>
<td>1940 (967,2725)</td>
<td>-</td>
</tr>
<tr>
<td><strong>LVEF (%)</strong></td>
<td>18% (15,28)</td>
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<td><strong>NYHA Class</strong></td>
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<td></td>
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<tr>
<td></td>
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* median (25%ile, 75%ile) of demographic data on heart failure patients and control group.

Samples were obtained from 14 patients and 16 controls.
Figure Legends

Figure 1. The corin-ANP system. Pro-ANP is cleaved by the serine protease corin to N-ANP and ANP. ANP activates natriuretic peptide receptor-A (NPR-A), a transmembrane guanylyl cyclase. NPR-A catalyzes the synthesis of cGMP, which mediates salt & water excretion, vasodilation and other effects.

Figure 2. Plasma levels of corin. Box plots showing corin concentrations (median, upper and lower quartiles) in plasma of individuals with acute heart failure (n = 14) and in pooled normal human plasmas (from 16 donors). p<0.01 by Mann Whitney analysis.

Figure 3. Pro-ANP cleavage in plasma from patients with acute heart failure in comparison with normal plasma. (A) The levels of N-terminal ANP-related peptides (pro-ANP + N-ANP) in the plasmas of individuals with acute heart failure and that of normal individuals (box plot showing median, upper and lower quartiles). (B) Depletion with C-ANP antibody but not control, non-specific antibody quantitatively removes C-ANP from samples. The mean level of C-ANP (ANP) peptide in samples after preincubation with anti-C-ANP antibody or with control antibody was determined by an immunoassay for C-ANP. (C) Depletion with C-ANP antibody does not affect N-ANP levels in samples. The mean level of N-ANP after preincubation with anti-C-ANP antibody or control antibody in assay buffer was determined by an immunoassay for N-ANP. Inset, the level of N-ANP after pre-incubation with anti-C-ANP or control antibody in plasma. (D) The levels of uncleaved pro-ANP in plasma of individuals with acute heart failure and in normal plasma (box plot showing median, upper and lower quartiles). (E)
Pro-ANP is detected by immunoblotting in heart failure plasma but not normal plasma. Pro-ANP was immunoprecipitated (IP) from heart failure or normal plasma by a pro-ANP antibody or no antibody and subjected to SDS-PAGE under reducing conditions. Membranes were immunoblotted (IB) with antibodies directed to the N-terminal sequence of pro-ANP (N-term), the C-terminal sequence of pro-ANP (C-term) or no antibody. Filled arrow identifies pro-ANP staining with both N-terminal and C-terminal antibodies; unfilled arrow identifies the light chain of the immunoprecipitating antibodies. Relative migration of molecular standards in kilo-Daltons is shown at right.

**Figure 4. Levels of cGMP and cleaved pro-ANP in patients with heart failure.** Box plot showing the median, upper and lower quartiles of cGMP level in plasma of individuals with heart failure and normal human plasma.
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