SUPPLEMENTAL METHODS

All human and animal experimental protocols used in the current study were approved by the Institutional Review Board and Animal Care and Use Committee at Mayo Clinic.

ProANP, ANP, BNP, and CNP synthesis and reagents

Recombinant human proANP for *ex vivo* and *in vitro* studies and recombinant canine proANP for *in vivo* canine studies were synthesized by ProMab Biotechnologies, Inc (Richmond, CA). Human proANP was tagged with Trx on the N-terminus and with 6-Histidine (His) on the C-terminus for peptide isolation (Fig. 5A for human proANP1-126), but canine proANP was untagged. ANP, canine BNP, and CNP for *in vitro* or *in vivo* studies were synthesized by Phoenix Laboratories, Inc (Burlingame, CA). The amino acid sequences of ANP and CNP are the same between human and canine.

In vivo studies in normal canines

We assessed the actions by intravenous bolus injection of equimolar doses (667 pmol/kg) (1) of canine proANP, ANP, canine BNP and placebo (0.9% saline) in normal adult male mongrel canines (21-30 kg, n=5 of each group). Acute procedures have been reported previously (2-4). Acute experiments permitted characterization of pharmacokinetics and cardiorenal function. The night before the acute experiment, the animals were fasted, given 300 mg lithium carbonate orally for assessment of renal tubular function, and allowed ad lib access to water. For the acute study, general anesthesia, endotracheal intubation and mechanical ventilation (ventilation rate 12/min; tidal volume 15 mL/kg; room air with 3 liters supplemental oxygen, Harvard Respirator) were performed. The right femoral artery was cannulated for direct mean

arterial pressure (MAP) measurement and arterial blood sampling. Electrocardiogram was continuously recorded for heart rate (HR). A Swan-Ganz catheter was inserted (Ohmedia; Criticath, WI) via the external jugular vein for measurement of right atrial pressure (RAP), pulmonary arterial pressure (PAP), pulmonary capillary wedge pressure (PCWP), cardiac output (CO), and body temperature. Systemic vascular resistance (SVR) was calculated as (MAP-RAP)/CO. Stroke volume (SV) was calculated as CO/HR. An arterial line was inserted into the femoral artery (for measurement of MAP and blood sampling) and two venous lines were inserted into the femoral vein (for inulin, saline/study drug injection). The ureter was cannulated via a left lateral flank for the timed urine collection and the renal artery was fitted with an electromagnetic flow probe to measure renal blood flow (RBF). All pressure data were continuously saved and analyzed by the mean value in iWorks LabScribe2 System (iWorks Inc., Dover, NH).

The study protocol started with a weight adjusted inulin bolus. Inulin was continuously infused at a rate of 1 mL/min for measurement of glomerular filtration rate (GFR). In addition, saline was infused at a rate of 1 mL/min. After an equilibration period of 60 min to stabilize inulin circulating concentration and hemodynamics and a 15 min baseline period, equimolar doses (667 pmol/kg) of canine proANP, ANP, BNP or placebo were injected (1). Hemodynamic measurements were continuously recorded, cardiac measurement and urine collection were performed at baseline, 5, 10, 20, 30, 60, 90, 120, 150, and 180 min after drug or placebo injection. From the arterial line, blood samples were drawn twice for baseline (= 0), 2, 4, 6, 8, 10, 15, 30, 45, 60, 90, 120, 150, and 180 min after peptide or placebo injection.

Urine and blood samples were collected on ice for volume, electrolytes and assays. Urine collected for cGMP analysis was heated to >90°C before storage. At the end of the experiment, the anesthetized animals were euthanized with an intravenous injection of pentobarbital.

Assays and electrolyte analysis

Cyclic GMP levels in cells, plasma or urine were assayed using a competitive RIA cGMP kit (Perkin-Elmer, Waltham, MA) (2-4). Plasma and urinary inulin concentrations were measured using anthrone methods. GFR was measured by inulin clearance. Plasma and urinary sodium (Na), potassium (K), and lithium (Li) levels were determined by flame emission spectrophotometry (Instrumentation Laboratory, Bedford, MA). Urinary excretion of each parameter (X), such as cGMP, Na or K were determined as X x urine volume (UV) (ml/min). Employing the lithium clearance (CL_{Li}) technique, we calculated proximal fractional reabsorption of sodium (PFR_{Na}) and distal fractional reabsorption of sodium (DFR_{Na}) according to the formula: $PFR_{Na} = [1 - (CL_{Li}/GFR)]$ x 100, $DFR_{Na} = [(CL_{Li} - CL_{Na})/CL_{Li}]$ x 100, in which $CL_{Li} = [(\text{urine Li x urine volume}) / \text{plasma Li}]$ and $CL_{Na} = [(\text{urine Na x urine volume}) / \text{plasma Na}]$ (3,4).

Pharmacokinetics

Pharmacokinetic profiles of proANP compared to ANP or BNP were calculated by the non-compartmental analysis using Phoenix WinNonline 6.3 Software (Pharsight, USA) (5).

In vitro study in HEK 293 cells

Human embryonic kidney (HEK) 293 cells were stably transfected with either human GC-A or GC-B using Lipofectamine (Invitrogen, Grand Island, NY) as previously described

(6,7). Transfected cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 250 ug/ml G418. Cells cultured in 6 well plates were incubated in HBSS (Invitrogen) containing 20 mmol/l HEPES, 0.1% BSA, and 0.5 mmol/l 3-isobutyl-1-methylxanthine (Sigma) with or without 10^{-10} to 10^{-8} M of proANP, ANP, BNP or CNP. The cGMP generation was stopped by ice-cold 6% TCA and then the treated cells were sonicated for 10 min. The samples were ether extracted four times in 4 volumes of ether, dried, and reconstituted in 300 µl cGMP assay buffer.

Blood sampling in healthy volunteers and patients with HF

Fresh serum was obtained from healthy volunteers (normals) of Olmsted County, Minnesota without cardiovascular, renal, pulmonary disease or diabetes and patients with HF, NYHA Class II-IV at admission to the emergency room or clinic, all of whom had informed consent. Blood samples were collected, and allowed to clot by leaving it undisturbed at room temperature. The clots were removed by centrifugation and serum were stored on ice and then examined.

Immunoprecipitation, Western blot, and sequencing of proANP in human serum ex vivo

Fresh serum samples (100 ul) with or without 500 ng of proANP (1.9×10^{-7}M) added were incubated for varying times from 5 to 180 min at 37°C as previously described (7,8). Following incubation, ANP molecular forms were immunoprecipitated using His-tag Ab coated Dynabeads (Invitrogen, Grand Island, NY). The protein immune-complexed beads were washed four times with Binding/Wash Buffer and then His-Elution Buffer was added. Immunoprecipitation pellets were heated at 95°C for 3 min in SDS sample buffer. For Western blot analysis, the immunoprecipitation beads were loaded onto polyacrylamide gels and

transferred onto PVDF membranes. Membranes were blocked, then incubated with rabbit polyclonal anti-6xHis Ab (1:3000, Abcam, Cambridge, MA). Membranes were washed in Tween-20 wash buffer and incubated with secondary Ab (1:10000, anti-rabbit Ab, Cell signaling Inc., Danvers, MA). Bands were visualized by ECL Prime (GE Healthcare Life Sciences, Pittsburgh, PA). Band intensity was quantified using the Fluor-S MultiImager (Bio-Rad, Hercules, CA). Protein bands were trypsin digested and sequenced using Edman sequencing by the Mayo Proteomics Core Facility (9).

Statistical analysis

Descriptive statistics are reported as mean ± SEM. Unpaired t-test was performed for comparison between groups. Comparisons within a group in time course of ex vivo and in vivo studies were made by 1-way ANOVA followed by the Bonferroni multiple comparison posttest analysis when the global test was significant. Two-way ANOVA was used to compare between groups in time course of in vivo study, followed by Bonferroni posttests. GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and JMP 10 were used for the above calculations. Statistical significance was accepted as $p<0.05$.

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SUPPLEMENTAL FIGURE LEDENDS

Supplemental Fig. 1. PreproANP amino acid sequences, processing and degradation.

This figure illustrates the cleavage of ANP signal peptide from preproANP, proANP processing into ANP (carperitide), and the degradation by Neprilysin (NEP) and insulin degrading enzyme (IDE).

Supplemental Fig. 2. *In vivo* plasma and urinary cGMP immunoreactivity and pharmacodynamics.

A: Plasma cGMP immunoreactivity, and D: Urinary cGMP excretion (UcGMPV). *$p<0.05$ vs baseline (= 0 min), †$p<0.05$ vs placebo, ‡$p<0.05$ vs ANP, and §$p<0.05$ vs BNP.

Supplemental Fig. 3. Summary.

Biological T½ is based on the half time from plasma pharmacokinetics of cGMP showed in Table 1. NEP, neprilysin; DPPIV, dipeptidyl peptidase-4; IDE, insulin degrading enzyme.

SUPPLEMENTAL TABLES

Supplemental Table 1. Baseline characteristics in normal canines.*

	ANP (n=5)	BNP (n=5)	ProANP (n=5)	Placebo (n=5)
Renal Function				
UV (ml/min)	0.14 ± 0.05	0.12 ± 0.04	0.19 ± 0.12	0.14 ± 0.03
UNaV (uEq/min)	16.7 ± 9.9	15.7 ± 4.8	12.2 ± 4.8	16.9 ± 3.4
UKV (uEq/min)	11.42 ± 2.26	8.03 ± 2.31	16.43 ± 5.52	10.07 ± 2.39
GFR (ml/min)	40.88 ± 5.78	33.75 ± 2.10	39.20 ± 4.70	34.66 ± 4.87
PFRNa (%)	85.83 ± 4.47	82.13 ± 2.77	86.77 ± 3.72	80.74 ± 2.16
DFRNa(%)	98.53 ± 0.54	98.37 ± 0.46	98.30 ± 0.63	98.16 ± 0.48
Hemodynamic Function				
HR (bpm)	90.5 ± 6.6	93.0 ± 5.6	109.5 ± 11.6	104.4 ± 6.3
MAP (mmHg)	113.2 ± 3.9	116.7 ± 4.0	117.3 ± 6.3	113.6 ± 1.7
PCWP (mmHg)	7.57 ± 0.98	6.79 ± 0.24	6.99 ± 0.59	5.93 ± 0.40
RAP (mmHg)	4.36 ± 1.43	2.99 ± 0.22	2.76 ± 0.56	2.89 ± 0.62
CO (l/min)	3.18 ± 0.26	2.64 ± 0.12	3.30 ± 0.64	3.39 ± 0.32
RBF (ml/min)	197.3 ± 53.4	194.7 ± 44.5	213.3 ± 29.7	176.2 ± 16.7
SV (ml/beats)	35.6 ± 3.3	28.7 ± 1.7	29.7 ± 3.0	32.4 ± 2.2
SVR (mmHg/L/min)	35.0 ± 2.53	43.6 ± 3.2	38.3 ± 5.43	34.0 ± 3.70
Hct (%)	39.1 ± 1.2	43.2 ± 2.0	37.4 ± 1.3	40.5 ± 2.6

UV, urine volume; UNaV, urinary sodium excretion; UKV, urinary potassium excretion; GFR, glomerular filtration rate; PFRNa, proximal fraction of sodium reabsorption; DFRNa, distal fraction of sodium reabsorption; HR, heart rate; MAP, mean arterial pressure; PCWP, pulmonary capirally wedge pressure; RAP, right atrial pressure; CO, cardiac output; RBF, renal blood flow; SV, stroke volume; SVR, systemic vascular resistance; and Hct, hematocrit. *There is no significant difference in any data between each group.

Supplemental Table 2. Changes of renal functions and cardiovascular hemodynamics; proANP compared with ANP or placebo.

Time (min)	5	10	20	30	45	60	90	120	150	180
UKV (uEq/min)										
Placebo	2.36 ± 0.74	1.44 ± 0.58	1.39 ± 0.15	2.73 ± 1.27	4.65 ± 2.11	5.32 ± 2.86	6.65 ± 1.78	**12.17 ± 2.69***	**16.34 ± 4.94***	**17.16 ± 4.22***
ANP	**31.21 ± 8.03*†**	16.78 ± 3.51	6.66 ± 1.11	5.10 ± 1.35	3.47 ± 1.93	5.79 ± 2.18	7.34 ± 3.38	13.63 ± 5.50	16.87 ± 6.05‡	**25.48 ± 9.02*‡**
BNP	**26.19 ± 7.13*†**	**28.39 ± 1.05*†**	**21.69 ± 4.20*†**	8.49 ± 3.19	4.70 ± 3.19	6.11 ± 1.84	1.97 ± 2.21	0.55 ± 1.90	0.43 ± 1.47	2.87 ± 1.91
ProANP	**41.89 ± 8.48*†**	**42.72 ± 5.91*†§**	**28.90 ± 2.83*†§**	9.85 ± 1.37	9.98 ± 2.16	**16.36 ± 2.16***	**18.30 ± 1.99*‡**	**18.88 ± 3.60*‡**	**17.97 ± 3.90*‡**	**18.28 ± 2.70***
GFR (ml/min)										
Placebo	8.96 ± 3.31	3.99 ± 5.87	0.54 ± 3.12	0.97 ± 2.21	8.48 ± 5.39	2.74 ± 4.87	6.22 ± 3.10	8.64 ± 1.67	5.44 ± 3.20	**17.24 ± 7.20***
ANP	**47.46 ± 12.59*†**	-9.40 ± 5.54‡	-5.35 ± 3.82	4.45 ± 4.43	-2.97 ± 6.76	-1.21 ± 10.2	-2.72 ± 6.04	-0.96 ± 7.63	10.78 ± 3.52	2.77 ± 5.20
BNP	**56.64 ± 10.21*†**	12.23 ± 4.11	4.75 ± 2.48	4.87 ± 5.21	14.77 ± 4.33	13.91 ± 4.79	13.06 ± 4.23	5.95 ± 2.13	10.58 ± 1.27	10.94 ± 0.97
ProANP	**75.84 ± 4.86*†§**	**15.94 ± 6.92*§**	11.61 ± 4.80	3.97 ± 3.57	**15.92 ± 2.04***	**15.55 ± 1.45***	**16.02 ± 2.59***	11.99 ± 2.86	13.31 ± 2.60	12.56 ± 2.78
PFRNa (%)										
Placebo	-4.09 ± 2.57	-2.58 ± 1.63	-5.22 ± 2.35	**-8.82 ± 2.10***	-6.22 ± 2.76	**-10.13 ± 2.22***	-8.06 ± 1.63	**-10.11 ± 2.06***	**-12.19 ± 2.58***	-8.04 ± 4.08
ANP	**-13.07 ± 2.04***	**-27.11 ± 6.15*†**	**-12.40 ± 3.10***	-11.10 ± 4.95	**-12.64 ± 4.12***	**-13.76 ± 4.16***	**-14.21 ± 3.66***	**-20.40 ± 6.44***	-11.85 ± 4.73	**-18.71 ± 4.89***
BNP	-6.98 ± 3.80	**-19.21 ± 6.41***	**-19.10 ± 4.27***	**-14.70 ± 5.46***	**-11.64 ± 4.55***	-10.12 ± 4.19	-5.93 ± 4.04	**-10.3 ± 6.08***	**-10.43 ± 6.47***	**-11.38 ± 5.41***
ProANP	-8.66 ± 1.56	**-30.12 ± 5.10*†**	**-26.12 ± 4.23*†**	**-22.07 ± 2.78***	**-20.47 ± 2.14***	**-26.11 ± 2.41***	**-25.88 ± 2.52*†§**	**-28.51 ± 5.49*†§**	**-25.93 ± 6.10***	**-23.35 ± 4.16***
DFRNa (%)										
Placebo	0.23 ± 0.17	0.10 ± 0.19	-0.02 ± 0.09	-0.07 ± 0.24	-0.26 ± 0.28	-0.14 ± 0.28	0.004 ± 0.20	-0.07 ± 0.11	-0.21 ± 0.19	-0.19 ± 0.17
ANP	**-3.35 ± 0.84*†**	**-4.77 ± 1.10*†§**	-1.71 ± 0.52§	-0.53 ± 0.34§	-0.23 ± 0.23	-0.20 ± 0.33	-0.42 ± 0.35	-0.43 ± 0.23	-0.47 ± 0.20	-0.62 ± 0.27
BNP	**-4.55 ± 0.42*†**	**-8.46 ± 1.03*†**	**-7.97 ± 1.69*†**	**-3.51 ± 0.91*†**	-1.71 ± 0.70	-0.96 ± 0.62	-0.45 ± 0.78	-0.61 ± 0.77	-0.99 ± 0.68	-1.48 ± 0.82
ProANP	**-4.40 ± 0.32*†**	**-9.27 ± 1.25*†‡**	**-9.01 ± 1.19*†‡**	**-4.05 ± 0.81*†‡**	**-2.66 ± 0.78***	**-3.14 ± 0.88*†‡**	**-2.62 ± 0.89***	**-2.34 ± 0.81***	**-1.85 ± 0.67***	-1.31 ± 0.58
RAP (mmHg)										
Placebo	-0.07 ± 0.08	-0.08 ± 0.09	-0.16 ± 0.12	-0.17 ± 0.11	-0.17 ± 0.14	-0.08 ± 0.14	0.10 ± 0.23	0.18 ± 0.19	0.32 ± 0.16	0.48 ± 0.31
ANP	-0.90 ± 0.20	-0.60 ± 0.13‡	-0.52 ± 0.15‡	-0.38 ± 0.22	-0.14 ± 0.14	-0.01 ± 0.17	-0.14 ± 0.27	0.07 ± 0.39‡	0.14 ± 0.59‡	0.27 ± 0.60‡
BNP	**-1.39 ± 0.15*†**	**-1.73 ± 0.16*†**	**-1.72 ± 0.15*†**	**-1.33 ± 0.13*†**	**-1.07 ± 0.10***	**-0.99 ± 0.10***	**-1.04 ± 0.03*†**	**-1.04 ± 0.12*†**	**-0.89 ± 0.18*†**	**-0.82 ± 0.17*†**
ProANP	-0.56 ± 0.13	-0.47 ± 0.19‡	**-0.86 ± 0.21***	**-1.44 ± 0.22*†§**	**-1.37 ± 0.27*†§**	**-1.30 ± 0.26*†§**	**-1.51 ± 0.29*†§**	**-1.28 ± 0.36*†§**	**-0.75 ± 0.36*†**	-0.06 ± 0.23
CO (l/min)										
Placebo	0.30 ± 0.18	0.31 ± 0.17	-0.03 ± 0.20	0.25 ± 0.17	0.08 ± 0.20	0.01 ± 0.20	-0.18 ± 0.21	-0.14 ± 0.34	-0.25 ± 0.42	-0.40 ± 0.48
ANP	0.44 ± 0.12‡	-0.03 ± 0.12‡	0.04 ± 0.16	0.05 ± 0.18	-0.05 ± 0.27	-0.10 ± 0.26	-0.26 ± 0.30	-0.37 ± 0.40	-0.28 ± 0.48	-0.34 ± 0.39
BNP	**2.32 ± 0.24*†**	**1.42 ± 0.21*†**	**0.66 ± 0.13***	0.39 ± 0.17	0.13 ± 0.07	0.22 ± 0.09	0.33 ± 0.16	0.44 ± 0.12	0.39 ± 0.12	0.48 ± 0.23
ProANP	**0.56 ± 0.18*‡**	0.31 ± 0.15‡	0.08 ± 0.18	0.27 ± 0.18	0.37 ± 0.22	**0.54 ± 0.19***	0.29 ± 0.08	-0.11 ± 0.08	-0.26 ± 0.17	**-0.61 ± 0.20*‡**
SV (ml/beats)										
Placebo	1.98 ± 0.76	0.56 ± 1.24	-1.39 ± 2.05	1.49 ± 1.34	0.18 ± 1.78	-0.17 ± 1.78	-2.17 ± 0.92	-1.92 ± 2.30	-3.28 ± 2.41	-4.36 ± 2.98
ANP	0.29 ± 0.95‡	-0.87 ± 1.13	0.53 ± 1.32	-0.20 ± 1.31	-0.64 ± 2.67	-0.72 ± 2.69	-4.10 ± 2.03	-6.03 ± 2.47	**-6.26 ± 4.59***	**-7.53 ± 3.69***

BNP	**8.69 ± 1.93***	3.39 ± 1.50	0.35 ± 1.43	1.72 ± 2.33	-0.49 ± 1.01	-0.22 ± 1.44	-0.98 ± 1.54	-0.91 ± 1.36	-1.80 ± 1.17	-0.87 ± 1.62
ProANP	1.19 ± 1.23	0.47 ± 0.45	-1.81 ± 1.03	**-3.57 ± 1.37***	**-3.35 ± 1.55***	**-4.04 ± 0.97***	**-6.65 ± 0.95***	**-8.56 ± 1.46*‡**	**-8.55 ± 0.92***	**-10.24 ± 0.91*‡**

GFR, glomerular filtration rate; RAP, right atrial pressure; CO, cardiac output; and SV, stroke volume. Data are expressed as mean ± SEM from

the each value of the difference from baseline levels. *$p<0.05$ vs baseline (=0 min); 1-way ANOVA with Bonferroni multiple-comparison test,

and †$p<0.05$ vs placebo, ‡$p<0.05$ vs ANP, and §$p<0.05$ vs BNP; 2-way ANOVA with Bonferroni multiple-comparison test.

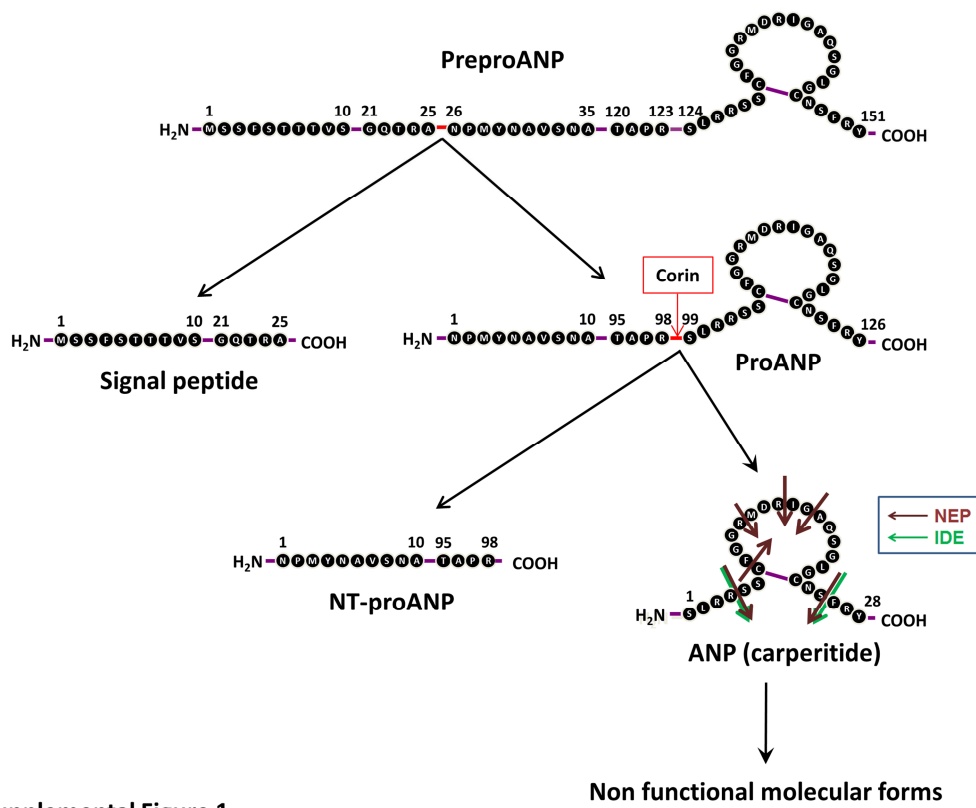**Supplemental Table 3. Characteristics of healthy subject vs patients with HF**.

	Normals (n=13)	Heart failure (n=14)	*p*
Age (yo)	41.2± 10.9	74.8 ± 3.1	<0.0001
Male (%)	61.5	85.7	0.21
NYHA II / III / IV	—	1/8/2005	—
EF (%)	—	43.9 ± 5.7	—
NT-proBNP (pg/ml)	38.4 ± 5.6	8511.9 ± 1885.2	0.0006
Cre (pg/ml)	0.82 ± 0.05	1.51 ± 0.12	<0.0001

Data are expressed as mean ± SEM. NYHA, New York Heart Association Class; EF, ejection fraction; NT-proBNP, amino-terminal proBNP; Cre, serum creatinine.
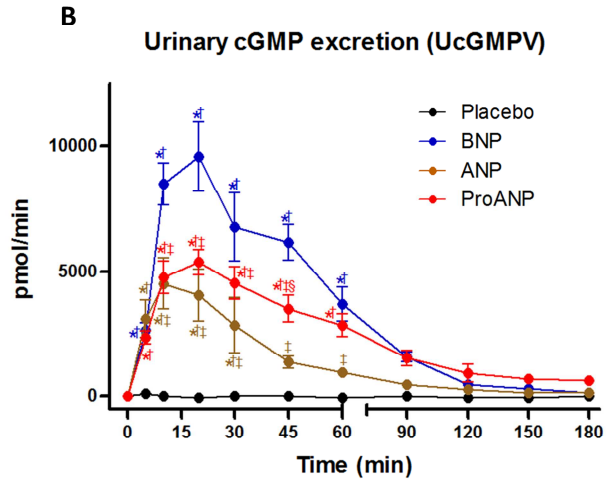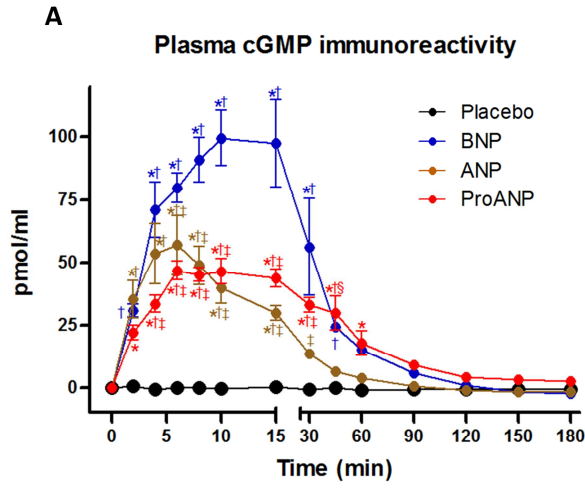
Supplemental Table 4. Individual characteristics of HF patients.

	Age (yo)	Gender	HF etiology	NYHA Class	EF (%)	Cre (mg/dl)	NT-proBNP (pg/ml)
1	77	M	HHD	4	74	2	3288
2	64	M	HCM	3	60	1.8	631
3	73	F	HCM, MR	4	51	1.6	12078
4	63	M	DCM	3	28	1.7	3142
5	85	M	MR	4	67	1.1	3547
6	63	M	HHD	3	55	1.4	2199
7	53	M	DCM	2	24	1.1	1034
8	73	M	ICM	3	30	1.6	7889
9	88	M	ICM	3	24	1.3	11740
10	83	M	ICM	4	20	1.8	20973
11	91	M	HHD	3	61	1.4	6313
12	68	M	CHD	4	27	2.5	13239
13	88	M	ICM	3	18	1	22590
14	78	F	MR	3	75	0.9	10503

HF, heart failure, NYHA, New York heart association; Cre, serum creatinine; NT-proBNP, N-terminal pro B-type natriuretic peptide; M, Male; F, Female; HHD, hypertensive heart disease; HCM, hypertrophic cardiomyopathy, MR, mitral valve regurgitation; DCM, dilated cardiomyopathy; ICM, Ischemic cardiomyopathy; CHD, congenital heart disease.
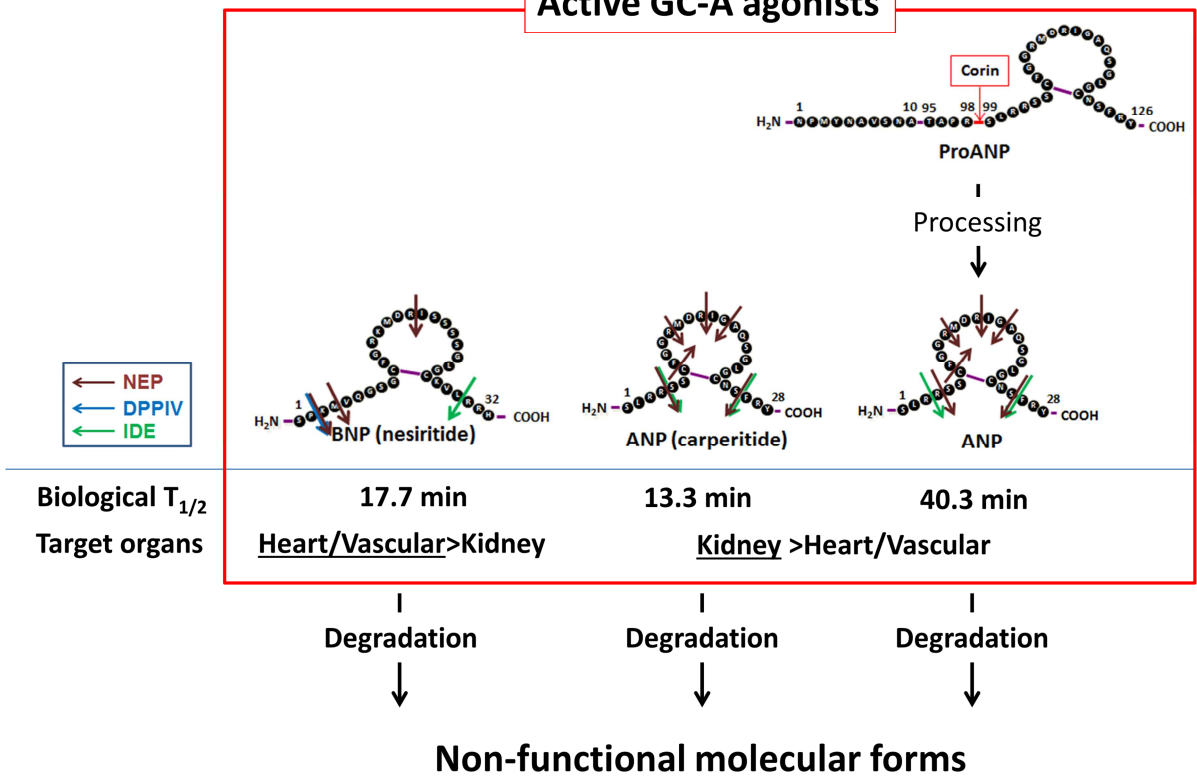
Supplemental Figure 1

A Plasma cGMP immunoreactivity

B Urinary cGMP excretion (UcGMPV)

Supplemental Figure 2

Active GC-A agonists

	BNP (nesiritide)	ANP (carperitide)	ANP
Biological T$_{1/2}$	17.7 min	13.3 min	40.3 min
Target organs	Heart/Vascular>Kidney	Kidney >Heart/Vascular	

Degradation → Non-functional molecular forms

Supplemental Figure 3