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Activation of a Novel Natriuretic Endocrine System in Humans with Heart Failure.

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Brief Title: Guanylin and Uroguanylin in Heart Failure

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Abstract

Objectives: To investigate whether plasma proguanylin and prouroguanylin levels are increased in patients with heart failure and evaluate their relationship with cardiac and renal function.

Background: Proguanylin and prouroguanylin are the inactive precursors of guanylin and uroguanylin, natriuretic peptides involved in the regulation of sodium balance. Urinary uroguanylin levels have previously been found to be elevated in patients with heart failure.

Methods & Results: In this prospective observational study we recruited 243 patients with heart failure (151 men), and 72 healthy controls. In heart failure patients, plasma levels of proguanylin (median 7.2 µg/L range [0.9 to 79.0]) and prouroguanylin (8.3 µg/L [1.7 to 53.0]) were both significantly higher compared to levels in healthy controls (5.5 µg/L [0.4 to 22.3]), $p < 0.0005$ and (6.3 µg/L [2.5 to 16.9]), $p < 0.0005$ respectively. In patients with heart failure, increased age, history of hypertension, diabetes, atrial fibrillation, use of diuretics, higher NYHA class and lower eGFR were significant univariate predictors of proguanylin and prouroguanylin level. In multivariate analysis a history of hypertension and low eGFR both had strong independent association with proguanylin and prouroguanylin level. Proguanylin and prouroguanylin varied significantly between NYHA class with a trend of increasing plasma concentration with worsening severity of symptoms.

Conclusions: Plasma proguanylin and prouroguanylin are elevated in patients with heart failure. Elevated plasma proguanylin and prouroguanylin levels are associated with hypertension, renal impairment and increasing severity of heart failure. This novel endocrine system may contribute to the pathophysiology of heart failure.

Introduction

Heart failure (HF) is associated with progressive impairment of sodium and water homeostasis [1]. Guanylin [2] and uroguanylin [3] are 15 and 16 amino acid peptides with natriuretic activity involved in the regulation of sodium balance in response to oral salt intake [4]. Both are synthesized as inactive precursors, proguanylin and prouroguanylin, which undergo post-secretory processing by proteolytic cleavage to produce their respective active moieties [5] [6].

Guanylin and uroguanylin produce their natriuretic effect by binding and activating cell surface guanylate cyclase C (GC-C) receptors located on gut mucosal and renal tubular epithelial cells initiating a cyclic guanosine monophosphate (cGMP) mediated second messenger cascade [7]. Guanylin and uroguanylin have also been shown to induce natriuresis in knockout mice lacking the GC-C receptor demonstrating a GC-C independent pathway [8].

A previous study demonstrated 70-fold increased uroguanylin-like bioactivity in the urine of patients with HF compared to healthy controls suggesting an important role of uroguanylin in the adaptive response to heart failure [9]. However this evidence was derived from bioassays, which lack the specificity of immunoassays and thus it was unclear whether urinary uroguanylin concentration in urine was elevated. Forte has suggested that uroguanylin may help regulate sodium balance via an intestinal-renal natriuretic axis [10] as it is observed that oral salt intake produces a more rapid natriuresis than an equivalent intravenous sodium load in healthy subjects [11].

We hypothesized that plasma proguanylin and prouroguanylin are elevated in HF as part of the counter-regulatory response to salt and water retention. Thus we measured the plasma concentrations of these peptides in patients admitted to hospital with HF and assessed the relationship of their levels to cardiac and renal function.

Methods

Study population

We included 243 consecutive patients referred to a tertiary referral cardiology centre in this study. This study abided by the Declaration of Helsinki and was approved by the local ethics committee. Written informed consent was obtained from all the patients. HF was diagnosed in patients fulfilling the following two diagnostic criteria: i) History of worsening or new onset shortness of breath and ii) Compatible clinical signs of pulmonary rales or elevated jugular venous pressure or peripheral oedema. Patients could already have a diagnosis of chronic HF or be a first presentation of acute HF. Exclusion criteria were known malignancy or surgery in the previous month. Renal function was assessed using plasma creatinine and estimated glomerular filtration rate (eGFR), calculated from the simplified formula derived from the Modification of Diet and Renal Disease (MDRD) study, validated in patients with heart failure [12].

Plasma samples

Blood samples were drawn on presentation with HF for determination of plasma levels of proguanylin and prouroguanylin. After 15 minutes of bed rest 20mL of blood was collected into tubes containing EDTA and aprotinin. All plasma was stored at -70°C until assayed in a blinded fashion in a single batch.

Echocardiography

Transthoracic echocardiography was performed in patients with a Sonos 5500 instrument (Philips Medical Systems, Reigate, UK). A 16-segment left ventricle (LV) wall-motion index based on the American Society of Echocardiography mode was derived by scoring each LV segment (1 = Normal, 2 = Hypokinesis, 3 = Akinesis) and dividing the total by the number of segments scored.

LV ejection fraction was calculated with the biplane method of discs formula. Impaired LV systolic function was defined as an LV ejection fraction $\leq 40\%$ or an LV wall-motion index ≥ 1.8 .

Proguanylin and prouroguanylin assays

These assays were performed using antibodies and standards from Biovendor, Brno, Czech Republic. Polyclonal antibodies to proguanylin or prouroguanylin were coated onto ELISA plates. Plasma samples (20 μ L for proguanylin and 50 μ L for prouroguanylin) were pipetted into the ELISA plate wells together with appropriate standards up to 10 or 20 ng/ml respectively). After incubation for 1 h at room temperature, the plates were washed, and then the conjugate 2nd polyclonal antibody pipetted into the wells for another 1 h of incubation. The prouroguanylin antibody was a biotinylated antibody, and the subsequent detection step used methyl-acridinium ester-labeled streptavidin on an MLX plate luminometer (Dynex Technologies Ltd, Worthing, UK). The proguanylin antibody was conjugated with horseradish peroxidase, and subsequent detection used a colorimetric substrate (3, 3', 5, 5'-tetramethylbenzidine) measured on a Dynex microplate reader using absorbance at 450nm. The lower limit of detection for proguanylin and prouroguanylin were 0.45 and 0.65 ng/ml respectively. The recoveries of proguanylin and prouroguanylin at 5 ng/ml were 93.6% and 101.8% respectively. Interassay coefficients of variation were 7.9% (at 13 ng/ml proguanylin, n=8) and 3.5% (at 2 ng/ml prouroguanylin, n=8). There was no cross reactivity of proguanylin in the prouroguanylin assay and vice versa.

Statistical analysis

Statistical analyses were performed on SPSS version 16 (SPSS Inc, Chicago, Ill). Between-group differences (healthy volunteers compared to patients with HF) in plasma proguanylin and prouroguanylin concentration and in demographic and clinical variables were compared using the Mann Whitney U test. Associations between plasma proguanylin and prouroguanylin levels and clinical variables were determined by Spearman correlations. The association between NYHA class and plasma proguanylin and prouroguanylin concentration was assessed using one way ANOVA of log transformed values with post hoc correction for multiple comparisons conducted using the Bonferroni correction. The ability of demographic factors and clinical variables to influence proguanylin and prouroguanylin level was assessed using the General Linear Model with log transformed plasma proguanylin or prouroguanylin concentration as the dependent variable. Factors significant in univariate analysis were entered as covariates into a multivariate model to identify independent predictors of plasma proguanylin and prouroguanylin concentration. Non-normally distributed variables are expressed as median [range]. A 2-tailed probability value of less than 0.05 was deemed to be statistically significant. All authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Demographic features of the HF and control populations are shown in Table 1. In the 72 healthy volunteers (median age 65.1 years [50 to 80]), there were no significant differences between plasma levels of proguanylin and prouroguanylin in males (5.7 μ g/L [0.4 to 13.0] and 6.1 μ g/L [2.5 to 16.9]) compared to females (5.7 μ g/L [3.3 to 22.3] and 7.2 μ g/L [3.5 to 12.6]) respectively. Plasma proguanylin levels were significantly greater in HF patients compared healthy controls (7.2 μ g/L [0.9 to 79.0] vs 5.5 μ g/L [0.4 to 22.3], $p < 0.0005$) as were prouroguanylin levels (8.3 μ g/L [1.7 to 53.0] vs 6.3 μ g/L [2.5 to 16.9], $p < 0.0005$). (Figure 1)

Univariate Analyses in HF Patients

Proguanylin and prouroguanylin levels showed significant univariate associations in those with previous history of hypertension, diabetes, atrial fibrillation, and prescription of diuretics. (Table 2) Plasma proguanylin and prouroguanylin levels were significantly inversely correlated with eGFR in

both healthy controls ($r = -0.329$, $p = 0.021$ and $r = -0.370$, $p = 0.009$ respectively) and patients with HF ($r = -0.430$, $p < 0.0005$ and $r = -0.455$, $p < 0.0005$ respectively). (Figures 2a & 2b) Plasma proguanylin was significantly correlated with increasing age in healthy controls ($r = 0.349$, $p = 0.003$) but not prouroguanylin ($r = 0.160$, $p = 0.178$) while both were in HF patients ($r = 0.380$, $p < 0.0005$ and $r = 0.323$, $p < 0.0005$) respectively. Plasma levels of proguanylin and prouroguanylin were strongly positively correlated with each other in both healthy controls ($r = 0.574$, $p < 0.0005$) and HF patients ($r = 0.771$, $p < 0.0005$).

There were no strong associations between proguanylin and prouroguanylin levels with echocardiographic parameters of LV structure and function. (Table 2)

HF – Variation with Severity

Plasma proguanylin and prouroguanylin both showed a general trend of increasing concentration with increasing NYHA class with significant variation between NYHA classes ($p < 0.0005$ for proguanylin and prouroguanylin). (Figure 3) In post hoc analysis plasma proguanylin levels in patients with NYHA class III HF (8.43 $\mu\text{g/L}$ [2.51 to 79.0]) and class IV HF (8.83 $\mu\text{g/L}$ [3.42 to 38.0]) were significantly greater than in healthy controls (5.69 $\mu\text{g/L}$ [0.42 to 22.3]), and those in class I HF (4.81 $\mu\text{g/L}$ [0.95 to 14.6]), all $p < 0.0005$. Similarly, plasma prouroguanylin levels were significantly greater in those in NYHA class III and IV HF compared to healthy controls (10.7 $\mu\text{g/L}$ [1.7 to 3.0] and 10.2 $\mu\text{g/L}$ [3.03 to 43.9] versus 6.32 $\mu\text{g/L}$ (2.53 to 21.7), $p < 0.0005$ and $p = 0.001$ respectively and compared to those in class I HF (6.46 $\mu\text{g/L}$ [1.84 to 26.6], $p = 0.002$ and $p = 0.014$ respectively).

Predictors of proguanylin and prouroguanylin in HF patients

In univariate general linear model analysis proguanylin and prouroguanylin levels varied significantly with increasing age, history of hypertension, diabetes, atrial fibrillation, diuretic use, NYHA class and eGFR. In multivariate general linear model analysis, the only independent predictors of proguanylin and prouroguanylin levels were history of hypertension and lower eGFR. (Tables 3 and 4)

Discussion

This study demonstrates that plasma proguanylin and prouroguanylin are elevated in patients with HF. We further showed that plasma proguanylin and prouroguanylin were independently associated with impaired renal function and hypertension with a trend of increasing plasma proguanylin and prouroguanylin levels with increasing severity of HF as measured by the NYHA class.

These results complement those of Carrithers *et al* [9] who found evidence of increased urinary uroguanylin bioactivity in patients with HF. Increased plasma proguanylin and prouroguanylin levels in HF are most likely as a result of impaired renal clearance leading to accumulation. Acute renal impairment has been demonstrated to reduce clearance of circulating prouroguanylin thus increasing its plasma levels [13]. Reduced glomerular filtration of plasma prouroguanylin would result in reduced delivery of the prohormone and less tubular conversion into its active form. However, local production of prouroguanylin in the kidney tubules with conversion to the active uroguanylin hormone may also contribute to the total uroguanylin-like activity in urine. It is unknown at present the extent of contribution of filtered prouroguanylin and tubular prouroguanylin to the urinary uroguanylin bioactivity in HF, and whether tubular production of the prohormone is upregulated in HF.

A further possibility is increased synthesis of proguanylin and prouroguanylin in patients with HF. The site of increased synthesis is most likely the intestine, as this has been shown to be the major source of circulating prouroguanylin that undergoes post secretory processing in the renal tubules [13][14]. The lack of significant correlation of plasma prouroguanylin and proguanylin levels with LV dimensions or left ventricle ejection fraction suggests that their synthesis may not be directly mediated by the pathophysiological processes involved in heart failure. Dieplinger *et al* has recently reported that both proguanylin and prouroguanylin have limited diagnostic utility as biomarkers for acute HF in patients presenting with acute dyspnoea [15] which indicates that long rather than short term processes may be responsible for elevations of their plasma levels.

Upregulation of guanylin and uroguanylin synthesis has been observed in the renal [16] and intestinal [17] tissues of rats fed a high salt diet. Changes in the intestinal mucosal microenvironment observed in heart failure as a result of tissue hypoperfusion and mesenteric ischaemia could potentially influence peptide synthesis [18][19].

Forte has hypothesised that prouroguanylin secreted from the intestine in response to oral salt loads acts as an endocrine hormone on the renal tubule epithelial cells as part of an intestinal-renal natriuretic axis [10]. However despite elevated plasma proguanylin and prouroguanylin and increased urinary bioactivity of uroguanylin, there remains a reduced natriuretic response to an oral salt load in subjects with HF compared to healthy subjects [1]. We propose there may be renal hyporesponsiveness to circulating plasma prouroguanylin, either from downregulation of uroguanylin receptor expression or altered receptor function. The combined effect of impaired renal response with reduced renal clearance of circulating prouroguanylin would result in diminished natriuretic and diuretic activity thus contributing to intravascular volume expansion. This may explain the correlation we found with elevated plasma pro-peptide levels and history of hypertension. However, our findings are hypotheses generating, and these possibilities remain to be investigated in prospectively designed studies.

Our study had several limitations. This was a small pilot study with a limited number of healthy controls and HF patients for comparison. Plasma guanylin, uroguanylin or urine uroguanylin were not measured since immunoassays specific for these small molecules are difficult to construct and are not currently available. However, measurement of these products may yield further information about the processing of these natriuretic prohormones that may be altered in heart failure in decompensated and treated scenarios.

In conclusion plasma levels of proguanylin and prouroguanylin are increased in patients with HF, particularly in symptomatic patients and those with renal impairment. These novel hormonal systems may contribute to the pathophysiology of salt homeostasis in heart failure.

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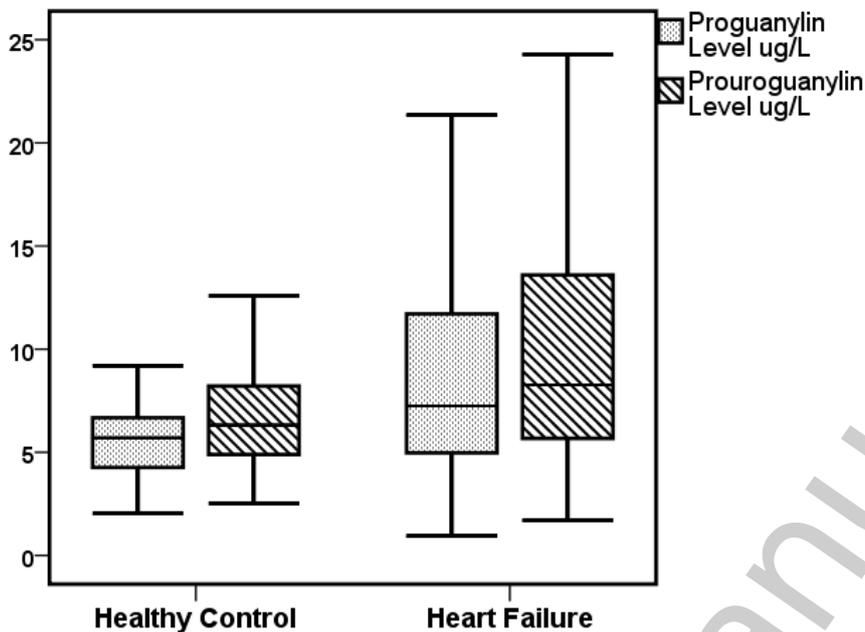
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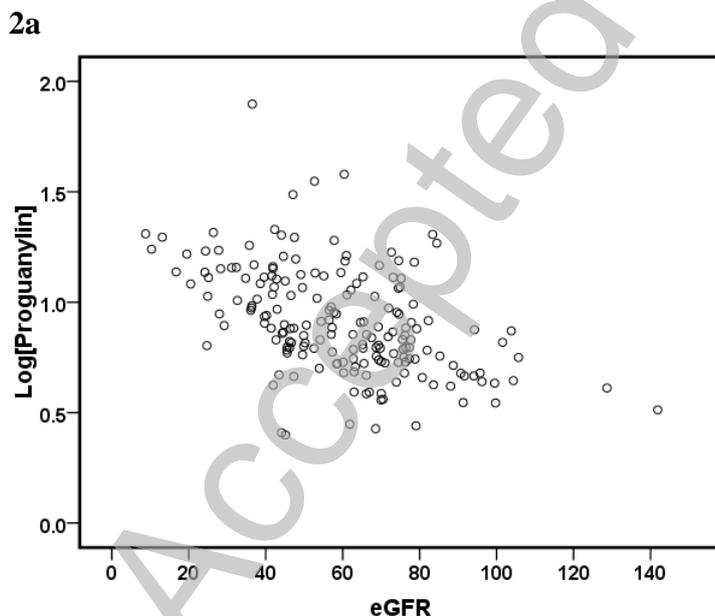
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Figure 1 Comparison of plasma proguanylin and prouroguanylin levels in healthy controls (n = 72) to subjects with heart failure (n = 243). Plasma proguanylin and prouroguanylin were both significantly elevated (p < 0.0005) in heart failure compared to healthy controls using the Mann Whitney U test.



Figures 2a & 2b Scatterplots illustrating relationship between plasma proguanylin and eGFR (Figure 2a) and plasma prouroguanylin and eGFR (Figure 2b) in HF patients. Spearman correlations $r = -0.430$, $p < 0.0005$ for proguanylin and eGFR and $r = -0.455$, $p < 0.0005$ for prouroguanylin and eGFR.



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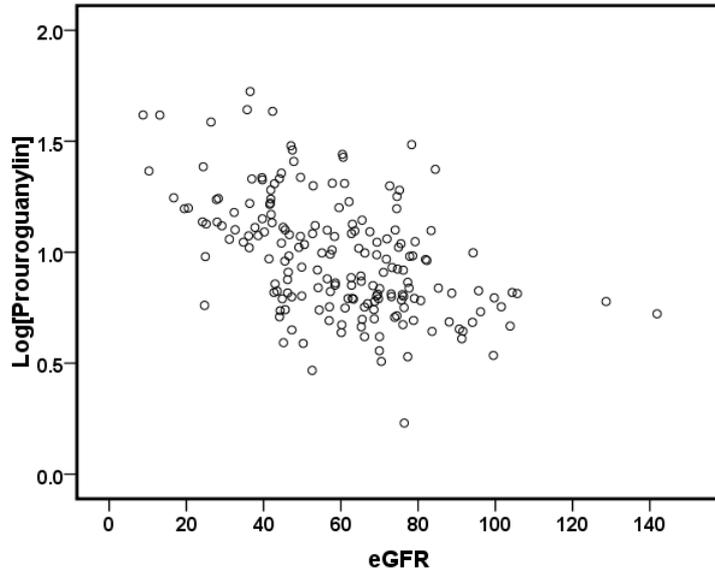
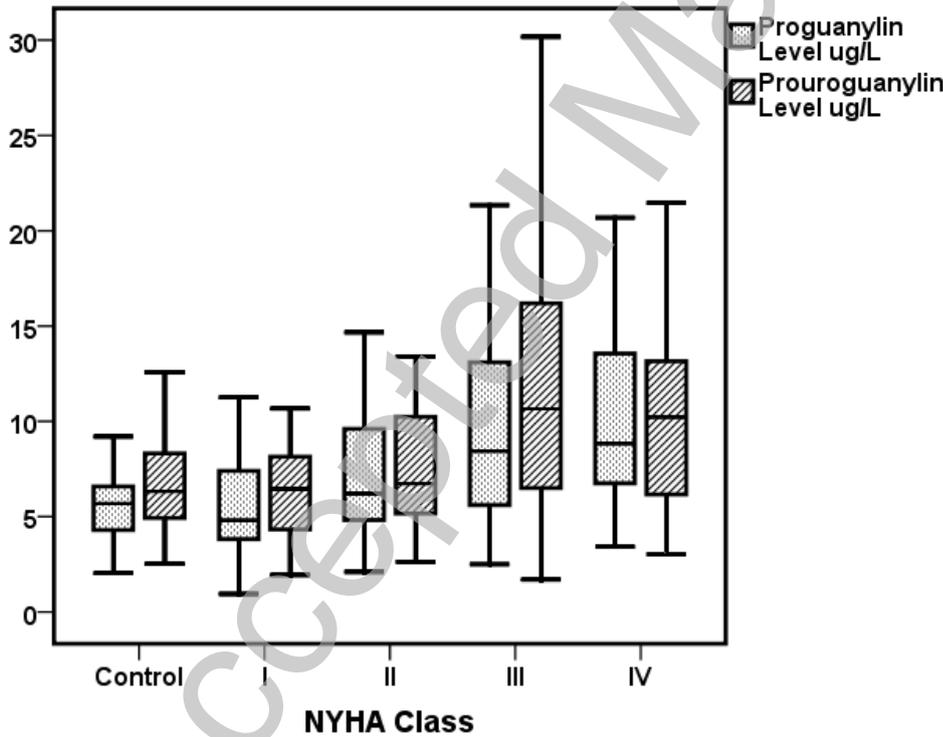


Figure 3 Comparison of plasma proguanylin and prouroguanylin levels between healthy control and different NYHA class. Both proguanylin and prouroguanylin showed a general trend of increasing level with increasing NHYA class with significant intergroup variation ($p < 0.0005$) using one way ANOVA.



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Table 1
Comparison between Healthy Controls and HF Patients

	Controls (n=72)	HF patients (n= 243)	p*
Age, y	65.1 (50 to 80)	70 (20 to 95)	0.014
Male Gender	43(59.7%)	151(62.1%)	
Proguanylin ug/L	5.5 (0.4 to 22.3)	7.2 (0.9 to 79.0)	<0.0005
Prouroguanylin ug/L	6.3 (2.5 to 16.9)	8.3 (1.7 to 53.0)	<0.0005
eGFR mLmin ⁻¹ 1.73m ⁻² surface area	71.8 (45.5 to 103.8)	50.6 (8.8 to 141.9)	<0.0005
LVEF	62.0 (51 to 77)	31.6 (15 to 45)	<0.0005
LVIDD	45.0 (30 to 56)	63.0 (40 to 95)	<0.0005
Past Medical History:			
Hypertension	None	109 (44.9)	
Myocardial infarction	None	92 (37.9)	
Diabetes Mellitus	None	43 (17.7)	
Medications:			
Diuretic	None	192 (79.0)	
Betablocker	None	97 (39.9)	
ACE inhibitor	None	163 (67.1)	
CaAntagonist	None	28 (11.5)	

Values are median (range) or n (%), * Mann Whitney U tests

LVEF = Left ventricular ejection fraction, LVIDD = Left ventricular internal diastolic dimension,

CaAntagonist = Calcium channel antagonist

Table 2
Univariate Analysis of Proguanylin and Prouroguanylin in HF Patients

	Proguanylin (ug/L)	p	Prouroguanylin (ug/L)	p
Males vs Females, median (range)	7.1 (0.94 to 79.0) vs 7.3 (2.5 to 20.7)	0.500	7.8 (1.7 to 53.0) vs 9.9 (2.9 to 43.9)	0.118
Previous medical history				
AMI vs none	7.2 (0.9 to 79.0) vs 7.9 (1.8 to 30.7)	0.890	8.3 (1.9 to 53.0) vs 9.2 (1.7 to 43.1)	0.941
Hypertension vs none	8.6 (2.1 to 38.0) vs 6.3 (0.9 to 79.0)	0.001	10.9 (1.7 to 43.9) vs 6.9 (1.9 to 53.0)	<0.0005
Diabetes vs none	9.9 (2.5 to 24.5) vs 7.0 (0.9 to 79.0)	0.004	11.2 (1.7 to 43.9) vs 8.1 (1.9 to 53.0)	0.012
Atrial Fibrillation vs none	10.3 (2.5 to 30.7) vs 6.7 (0.9 to 79.0)	0.001	11.8 (3.9 to 43.8) vs 8.1 (1.7 to 53.0)	0.002
Drug History				
Diuretic vs none	8.2 (0.9 to 79.0) vs 5.2 (3.0 to 18.5)	<0.0005	9.6 (1.7 to 53.0) vs 6.5 (2.8 to 26.6)	0.003
Betablocker vs none	7.1 (0.9 to 38.0) vs 7.6 (1.8 to 79.0)	0.218	7.1 (1.9 to 43.9) vs 9.26 (1.7 to 53.0)	0.217
ACEi vs none	7.8 (1.8 to 79.0) vs 6.8 (0.9 to 35.3)	0.100	8.6 (1.7 to 53.0) vs 6.9 (1.9 to 41.5)	0.168
CaAntagonist vs none	5.4 (0.9 to 20.4) vs 6.3 (1.8 to 79.0)	0.958	6.6 (1.9 to 41.5) vs 7.6 (1.7 to 53.0)	0.669
Spearman correlations				
Age	0.380	<0.0005	0.323	<0.0005
eGFR	-0.430	<0.0005	-0.455	<0.0005
Creatinine	0.514	<0.0005	0.455	<0.0005
NYHA class	0.354	<0.0005	0.242	<0.0005
LVIDD	-0.066	0.525	-0.095	0.363
LVEF	0.154	0.159	-0.030	0.784

AMI = Acute myocardial infarction, CaAntagonist = Calcium channel antagonist, NYHA = New York Heart Association, LVIDD = Left ventricular internal diastolic dimension, LVEF = Left ventricular ejection fraction

Table 3
Predictors of Plasma Proguanylin* Level in HF patients

	Univariate			Multivariate		
	B	95% C.I.	p	B	95% C.I.	p
Age	0.01	(0 to 0.01)	<0.00005	0	(-0.01 to 0)	0.511
Sex	0.01	(-0.06 to 0.08)	0.750			
Past Medical History						
MI	0	(-0.08 to 0.07)	0.930			
Hypertension	-0.11	(-0.18 to -0.05)	0.001	-0.31	(-0.61 to -0.01)	0.045
Diabetes	-0.12	(-0.20 to -0.03)	0.010	-0.04	(-0.23 to 0.14)	0.637
Atrial Fibrillation	-0.12	(-0.21 to -0.04)	0.003	-0.07	(-0.26 to 0.12)	0.481
Medication History						
Diuretic	-0.17	(-0.25 to -0.09)	<0.00005	-0.05	(-0.65 to 0.74)	0.897
Beta Blocker	0.05	(-0.16 to 0.12)	0.131			
ACEi	-0.06	(-0.14 to 0.01)	0.078			
Calcium Antagonist	0.02	(-0.09 to 0.13)	0.737			
LVIDD	0	(-0.01 to 0)	0.659			
NYHA	0.09	(0.06 to 0.12)	<0.00005	0.05	(-0.01 to 0.11)	0.083
eGFR	-0.01	(-0.01 to 0)	<0.00005	0	(-0.01 to 0)	<0.00005

* Log transformed, MI = Myocardial Infarction, LVIDD = Left Ventricular Internal Diastolic Dimension
C.I.= Confidence Interval, B = Linear Regression Coefficient

Table 4
Predictors of Plasma Prouroguanylin* Level in HF patients

	Univariate			Multivariate		
	B	95% C.I.	p	B	95% C.I.	p
Age	0.01	(0 to 0.01)	<0.00005	0	(-0.01 to 0)	0.630
Sex	0.06	(-0.02 to 0.13)	0.142			
Past Medical History						
MI	-0.01	(-0.07 to 0.09)	0.854			
Hypertension	-0.14	(-0.21 to -0.07)	<0.00005	-0.39	(-0.72 to -0.06)	0.021
Diabetes	-0.11	(-0.20 to -0.02)	0.021	-0.01	(-0.20 to 0.19)	0.959
Atrial Fibrillation	-0.14	(-0.23 to -0.05)	0.002	-0.12	(-0.34 to 0.09)	0.246
Medication History						
Diuretic	-0.13	(-0.21 to -0.04)	0.005	0.12	(-0.63 to 0.88)	0.747
Beta Blocker	0.04	(-0.03 to 0.11)	0.282			
ACEi	-0.06	(-0.13 to -0.02)	0.156			
Calcium Antagonist	-0.01	(-0.10 to 0.12)	0.916			
LVIDD	0	(-0.01 to 0)	0.333			
NYHA	0.07	(0.03 to 0.10)	<0.00005	0.02	(-0.04 to 0.09)	0.501
eGFR	-0.01	(-0.01 to -0)	<0.00005	-0.01	(-0.01 to 0.0)	<0.00005

* Log transformed, MI = Myocardial Infarction, LVIDD = Left Ventricular Internal Diastolic Dimension
C.I.= Confidence Interval, B = Linear Regression Coefficient