

# Activation of a novel natriuretic endocrine system in humans with heart failure

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## A B S T R A C T

Proguanylin and prouroguanylin are the inactive precursors of guanylin and uroguanylin, natriuretic peptides involved in the regulation of sodium balance. Urinary uroguanylin levels have been found previously to be elevated in patients with HF (heart failure). The aim of the present study was to investigate whether plasma proguanylin and prouroguanylin levels are increased in patients with HF and to evaluate their relationship with cardiac and renal function. In this prospective observational study, we recruited 243 patients with HF (151 men) and 72 healthy controls. In patients with HF, plasma levels of proguanylin [median, 7.2 (range, 0.9–79.0)  $\mu\text{g/l}$ ] and prouroguanylin [8.3 (1.7–53.0  $\mu\text{g/l}$ )] were both significantly ( $P < 0.0005$ ) higher compared with levels in healthy controls [5.5 (0.4–22.3  $\mu\text{g/l}$ ) for proguanylin and 6.3 (2.5–16.9)  $\mu\text{g/l}$  for prouroguanylin]. In patients with HF, increased age, a history of hypertension, diabetes and atrial fibrillation, use of diuretics, a higher NYHA (New York Heart Association) class and a lower eGFR (estimated glomerular filtration rate) were significant univariate predictors of proguanylin and prouroguanylin levels. In multivariate analysis, a history of hypertension and low eGFR both had strong independent associations with proguanylin and prouroguanylin levels. Proguanylin and prouroguanylin varied significantly between NYHA class with a trend of increasing plasma concentrations with worsening severity of symptoms. In conclusion, plasma proguanylin and prouroguanylin are elevated in patients with HF. Elevated plasma proguanylin and prouroguanylin levels are associated with hypertension, renal impairment and increasing severity of HF. This novel endocrine system may contribute to the pathophysiology of HF.

## INTRODUCTION

HF (heart failure) 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 GC-C (guanylate cyclase C) receptors located on gut mucosal and renal tubular epithelial cells initiating a 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 a 70-fold increase in uroguanylin-like bioactivity in the urine of patients with HF compared with healthy controls, suggesting an

**Key words:** heart failure, natriuretic peptide, hypertension, proguanylin, prouroguanylin, sodium balance.

**Abbreviations:** GC-C, guanylate cyclase C; eGFR, estimated glomerular filtration rate; HF, heart failure; LV, left ventricular; LVEF, LV ejection fraction; LVWMI, LV wall motion index; NYHA, New York Heart Association.

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important role of uroguanylin in the adaptive response to HF [9]. However, this evidence was derived from bioassays, which lack the specificity of immunoassays and, thus, it was unclear whether urinary uroguanylin concentration was elevated. Forte [10] has suggested that uroguanylin may help regulate sodium balance via an intestinal–renal natriuretic axis, 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 with cardiac and renal function.

## MATERIALS AND METHODS

### Study population

We included 243 consecutive patients referred to a tertiary referral cardiology centre in the present study. The present study abided by the Declaration of Helsinki and was approved by the local ethics committee. Written informed consent was obtained from all of the patients.

HF was diagnosed in patients fulfilling the following two diagnostic criteria: (i) a 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 eGFR (estimated glomerular filtration rate), calculated from the simplified formula derived from the MDRD (Modification of Diet and Renal Disease) study, validated in patients with HF [12].

### Plasma samples

Blood samples were drawn on presentation with HF for determination of plasma levels of proguanylin and prouroguanylin. After 15 min of bed rest, 20 ml of blood was collected into tubes containing EDTA and aprotinin. All plasma was stored at  $-70^{\circ}\text{C}$  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). A 16-segment LVWMI [LV (left ventricular) wall motion index] based on the American Society of Echocardiography mode was derived by scoring each left ventricle segment (1 = normal; 2 = hypokinesis; and

3 = akinesis) and dividing the total by the number of segments scored. LVEF (LV ejection fraction) was calculated with the biplane method of discs formula. Impaired LV systolic function was defined as an LVEF  $\leq 40\%$  or an LVWMI  $\geq 1.8$ .

### Proguanylin and prouroguanylin assays

These assays were performed using antibodies and standards from Biovendor. Polyclonal antibodies to proguanylin or prouroguanylin were coated on to ELISA plates. Plasma samples (20  $\mu\text{l}$  for proguanylin and 50  $\mu\text{l}$  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 ( $25^{\circ}\text{C}$ ), the plates were washed and then the conjugated secondary polyclonal antibody was pipetted into the wells for incubation for another 1 h. The prouroguanylin antibody was a biotinylated antibody, and the subsequent detection step used methyl-acridinium ester-labelled streptavidin on an MLX plate luminometer (Dynex Technologies). The proguanylin antibody was conjugated to horseradish peroxidase, and subsequent detection used a colorimetric substrate (3,3',5,5'-tetramethylbenzidine) measured on a Dynex microplate reader with absorbance at 450 nm. The lower limits 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. Inter-assay 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. Between-group differences (healthy volunteers compared with patients with HF) in plasma proguanylin and prouroguanylin concentrations and in demographics 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 (New York Heart Association) class and plasma proguanylin and prouroguanylin concentrations 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 levels was assessed using the General Linear Model with log-transformed plasma proguanylin or prouroguanylin concentrations 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 concentrations.

**Table 1** Comparison between healthy controls and patients with HF

Values are medians (range) or numbers (percentage). *P* values were determined using a Mann–Whitney *U* test. ACEI, angiotensin-converting enzyme inhibitor; CaAntagonist, calcium channel antagonist; LVIDD, LV internal diastolic dimension; MI, myocardial infarction.

Characteristic	Controls ( <i>n</i> = 72)	HF patients ( <i>n</i> = 243)	<i>P</i> value
Age (years)	65.1 (50 to 80)	70 (20–95)	0.014
Male gender ( <i>n</i> )	43 (59.7 %)	151 (62.1 %)	
Proguanylin (μg/l)	5.5 (0.4–22.3)	7.2 (0.9–79.0)	< 0.0005
Prouroguanylin (μg/l)	6.3 (2.5–16.9)	8.3 (1.7–53.0)	< 0.0005
eGFR (ml · min <sup>-1</sup> · 1.73 m <sup>-2</sup> surface area)	71.8 (45.5–103.8)	50.6 (8.8–141.9)	< 0.0005
LVEF (%)	62.0 (51–77)	31.6 (15–45)	< 0.0005
LVIDD (mm)	45.0 (30–56)	63.0 (40–95)	< 0.0005
Past medical history ( <i>n</i> )			
Hypertension	None	109 (44.9)	
MI	None	92 (37.9)	
Diabetes	None	43 (17.7)	
Medication ( <i>n</i> )			
Diuretic	None	192 (79.0)	
β-Blocker	None	97 (39.9)	
ACEI	None	163 (67.1)	
CaAntagonist	None	28 (11.5)	

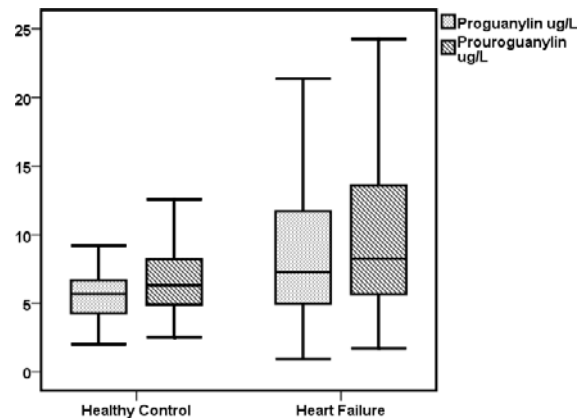
Non-normally distributed variables are expressed as medians (range). A two-tailed *P* value of less than 0.05 was deemed to be statistically significant.

## RESULTS

Demographic features of the HF and control populations are shown in Table 1. In the 72 healthy volunteers, there were no significant differences between plasma levels of proguanylin and prouroguanylin in males [5.7 (0.4–13.0) μg/l and 6.1 (2.5–16.9) μg/l respectively] and females [5.7 (3.3–22.3) μg/l and 7.2 (3.5–12.6) μg/l respectively]. Plasma proguanylin levels were significantly (*P* < 0.0005) greater in patients with HF compared with healthy controls as were prouroguanylin levels (Table 1 and Figure 1).

### Univariate analyses in patients with HF

Proguanylin and prouroguanylin levels had significant univariate associations in those patients with a previous history of hypertension, diabetes, atrial fibrillation and a 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) (Figure 2). 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), whereas both were significantly correlated in patients with HF (*r* = 0.380,



**Figure 1** Comparison of plasma proguanylin and prouroguanylin levels in healthy controls (*n* = 72) with patients with HF (*n* = 243)

Plasma proguanylin and prouroguanylin were both significantly elevated (*P* < 0.0005) in HF compared with healthy controls, as determined using the Mann–Whitney *U* test.

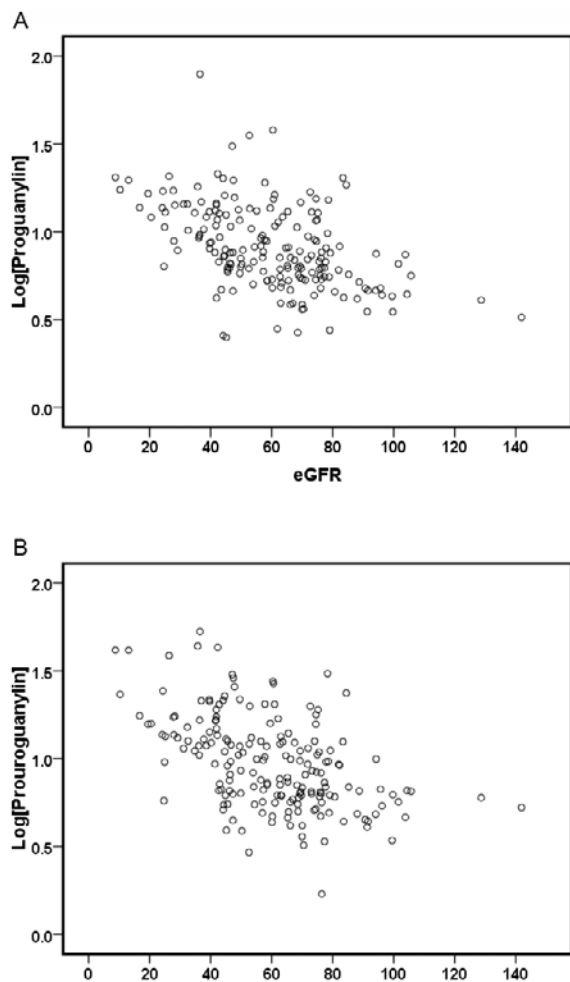
*P* < 0.0005, and *r* = 0.323, *P* < 0.0005 respectively). Plasma levels of proguanylin and prouroguanylin were strongly positively correlated with each other in both healthy controls (*r* = 0.574, *P* < 0.0005) and patients with HF (*r* = 0.771, *P* < 0.0005).

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

**Table 2** Univariate analysis of proguanylin and prouroguanylin in patients with HF

Values are medians (range). ACEI, angiotensin-converting enzyme inhibitor; AMI, acute myocardial infarction; CaAntagonist, calcium channel antagonist; LVIDD, LV internal diastolic dimension.

Analysis	Proguanylin ( $\mu\text{g/l}$ )	<i>P</i> value	Prouroguanylin ( $\mu\text{g/l}$ )	<i>P</i> value
Males compared with females	7.1 (0.94 – 79.0) compared with 7.3 (2.5–20.7)	0.500	7.8 (1.7–53.0) compared with 9.9 (2.9–43.9)	0.118
Previous medical history				
AMI compared with none	7.2 (0.9–79.0) compared with 7.9 (1.8–30.7)	0.890	8.3 (1.9–53.0) compared with 9.2 (1.7–43.1)	0.941
Hypertension compared with none	8.6 (2.1–38.0) compared with 6.3 (0.9–79.0)	0.001	10.9 (1.7–43.9) compared with 6.9 (1.9–53.0)	<0.0005
Diabetes compared with none	9.9 (2.5–24.5) compared with 7.0 (0.9–79.0)	0.004	11.2 (1.7–43.9) compared with 8.1 (1.9–53.0)	0.012
Atrial fibrillation compared with none	10.3 (2.5–30.7) compared with 6.7 (0.9–79.0)	0.001	11.8 (3.9–43.8) compared with 8.1 (1.7–53.0)	0.002
Drug history				
Diuretic compared with none	8.2 (0.9–79.0) compared with 5.2 (3.0–18.5)	< 0.0005	9.6 (1.7–53.0) compared with 6.5 (2.8–26.6)	0.003
$\beta$ -Blocker compared with none	7.1 (0.9–38.0) compared with 7.6 (1.8–79.0)	0.218	7.1 (1.9–43.9) compared with 9.26 (1.7–53.0)	0.217
ACEI compared with none	7.8 (1.8–79.0) compared with 6.8 (0.9–35.3)	0.100	8.6 (1.7–53.0) compared with 6.9 (1.9–41.5)	0.168
CaAntagonist compared with none	5.4 (0.9–20.4) compared with 6.3 (1.8–79.0)	0.958	6.6 (1.9–41.5) compared with 7.6 (1.7–53.0)	0.669
Spearman correlation				
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

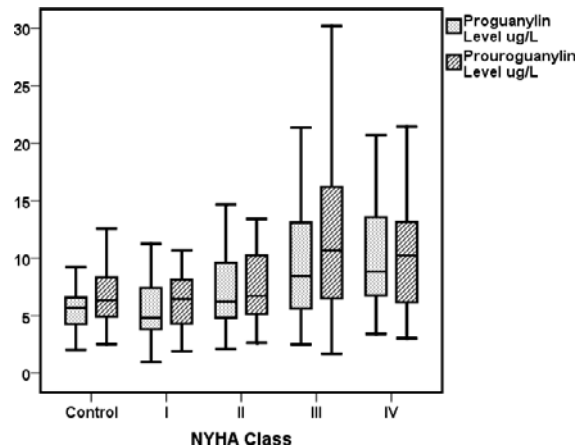


**Figure 2** Relationship between eGFR and plasma proguanylin (a) and prouroguanylin (b) in patients with HF

Spearman correlations:  $r = -0.430$ ,  $P < 0.0005$  for proguanylin and eGFR, and  $r = -0.455$ ,  $P < 0.0005$  for prouroguanylin and eGFR.

### HF: variation with severity

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



**Figure 3** Comparison of plasma proguanylin and prouroguanylin levels between healthy controls and patients in different NYHA classes

Both proguanylin and prouroguanylin had a general trend of increasing levels with increasing NYHA class, with significant inter-group variation ( $P < 0.0005$ ) using one-way ANOVA.

### Predictors of proguanylin and prouroguanylin in patients with HF

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

### DISCUSSION

The results of the present study demonstrate that plasma proguanylin and prouroguanylin levels are elevated in patients with HF. We have shown further that plasma proguanylin and prouroguanylin levels 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 the clearance of circulating prouroguanylin, thus increasing its plasma levels [13]. Decreased glomerular filtration of plasma prouroguanylin would result in reduced delivery of the

**Table 3 Predictors of plasma proguanylin level in patients with HF**

Plasma proguanylin levels were log-transformed. ACEI, angiotensin-converting enzyme inhibitor; *B*, linear regression coefficient; CaAntagonist, calcium channel antagonist; CI, confidence interval; LVDD, LV internal diastolic dimension; MI, myocardial infarction.

Predictor	Univariate			Multivariate		
	<i>B</i>	95 % CI	<i>P</i> value	<i>B</i>	95 % CI	<i>P</i> value
Age	0.01	0 to 0.01	< 0.00005	0	− 0.01 to 0	0.511
Gender	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			
CaAntagonist	0.02	− 0.09 to 0.13	0.737			
LVDD	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

**Table 4 Predictors of plasma prouroguanylin level in patients with HF**

Plasma prouroguanylin levels were log-transformed. ACEI, angiotensin-converting enzyme inhibitor; *B*, linear regression coefficient; CaAntagonist, calcium channel antagonist; CI, confidence interval; LVDD, LV internal diastolic dimension; MI, myocardial infarction.

Predictor	Univariate			Multivariate		
	<i>B</i>	95 % CI	<i>P</i> value	<i>B</i>	95 % CI	<i>P</i> value
Age	0.01	0 to 0.01	< 0.00005	0	− 0.01 to 0	0.630
Gender	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			
CaAntagonist	− 0.01	− 0.10 to 0.12	0.916			
LVDD	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

prohormone and less tubular conversion into its active form.

However, local production of prouroguanylin in the kidney tubules with conversion into the active uroguanylin hormone may also contribute to the total uroguanylin-like activity in urine. It is unknown at present the extent of the contribution of filtered prouroguanylin and tubular prouroguanylin to the

urinary uroguanylin bioactivity in HF, and whether tubular production of the prohormone is up-regulated in HF.

A further possibility is the increased synthesis of proguanylin and prouroguanylin in patients with HF. The site of increased synthesis is most probably 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 a significant correlation between plasma prouroguanylin and proguanylin levels with LV dimensions or LVEF suggests that their synthesis may not be directly mediated by the pathophysiological processes involved in HF. Dieplinger et al. [15] have reported recently that both proguanylin and prouroguanylin have limited diagnostic utility as biomarkers for acute HF in patients presenting with acute dyspnoea, which indicates that long- rather than short-term processes may be responsible for elevations of their plasma levels.

Up-regulation 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 HF as a result of tissue hypoperfusion and mesenteric ischaemia could potentially influence peptide synthesis [18,19].

Forte [10] has hypothesized 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. 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 with healthy subjects [1]. We propose there may be renal hyporesponsiveness to circulating plasma prouroguanylin, either from down-regulation 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 observed with elevated plasma pro-peptide levels and a history of hypertension. However, our findings are hypotheses-generating, and these possibilities remain to be investigated in prospectively designed studies.

Our present study had several limitations. This was a small pilot study with a limited number of healthy controls and patients with HF 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 HF 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 HF.

## FUNDING

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