Guanylin peptides in heart failure

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by

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Abstract

This study investigated the role of prouroguanylin (ProUGN) and proguanylin (ProGN), members of a novel class of peptides with natriuretic activity in heart failure (HF), a disorder of declining cardiac output associated with disturbed sodium and water homeostasis. The hypothesis was that ProUGN and ProGN activity is dysregulated in chronic and acute HF.

Plasma ProUGN and ProGN were measured in 243 patients with chronic stable HF and plasma ProUGN and cGMP, an intracellular mediator of ProUGN activity, measured in 336 patients admitted to hospital with acute HF using immunoassays. ProUGN and cGMP levels were repeated in acute HF patients prior to discharge. The primary endpoints were all cause mortality, HF readmission and either outcome at 180 days.

ProUGN and ProGN were significantly greater in patients with chronic HF compared to controls and inversely correlated with eGFR. ProUGN and ProGN were significantly greater in patients with hypertension and in those taking diuretics, with higher levels associated with increased severity of HF as assessed by NYHA class. In multivariate analysis, eGFR was the only independent predictor of plasma ProUGN and ProGN level.

ProUGN and cGMP were significantly lower in patients with acute HF compared to in controls. Pre-discharge ProUGN and cGMP were significantly greater than at admission, with pre-discharge ProUGN significantly greater than in controls. Admission ProUGN was significantly greater in patients who died and a greater pre-discharge ProUGN was significantly associated with increased risk of early mortality. Pre-discharge cGMP levels were significantly lower in those readmitted with HF compared to those not, with higher levels significantly associated with reduced risk of early HF readmission. A greater pre-discharge ProUGN/cGMP ratio was significantly associated with increased risk of mortality or HF readmission. These results suggest that adverse outcomes in HF may be associated with hyporesponsiveness to ProUGN.

Acknowledgements

I personally recruited, collected and analysed blood samples and undertook echocardiography for the majority of patients in the acute heart failure cohort of the study. Dr Turab Ali recruited, collected blood samples and undertook echocardiography in the chronic heart failure cohort of the study. I performed statistical analysis on all data and am solely responsible for the authorship of this manuscript.

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Publications relating to this thesis

Narayan H, Mohammed N, Quinn PA, Squire IB, Davies JE, Ng LL. Activation of a novel natriuretic endocrine system in humans with heart failure. *Clin Sci* 2010;118:367-74

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List of Abbreviations

ACEi	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndrome
AF	Atrial fibrillation
AHFS	Acute HF syndromes
ANP	Atrial natriuretic peptide
ARA	Aldosterone receptor antagonist
ARB	Angiotensin receptor blocker
AUC	Area under curve
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
CCB	Calcium channel blocker
CCD	Cortical collecting duct
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
CXR	Chest X Ray
DM	Diabetes mellitus
ECG	Electrocardiogram
ECHO	Echocardiogram
EF	Ejection fraction
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
GC	Guanylate cyclase
GC-C	Guanylate cyclase C
GFR	Glomerular filtration rate
GN	Guanylin
HF	Heart failure
HFNEF	Heart failure with normal ejection fraction
HFPEF	Heart failure with preserved ejection fraction
HPLC	High performance liquid chromatography
HR	Hazard ratio
HTN	Hypertension

IHD	Ischaemic heart disease
IQR	Interquartile range
JVP	Jugular venous pressure
LV	Left ventricle
LVIDD	Left ventricle internal diameter in diastole
LVIDS	Left ventricle internal diameter in systole
LVSD	Left ventricle systolic dysfunction
MAE	Methyl acridinium ester
MI	Myocardial infarction
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NMR	Nuclear magnetic resonance
NPR-A	Natriuretic peptide receptor-A
NPV	Negative predictive value
NTproBNP	N-Terminal pro-BNP
NYHA	New York Heart Association
OR	Odds ratio
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PKG	cGMP dependent protein kinase
PND	Paroxysmal nocturnal dyspnoea
PPV	Positive predictive value
ProGN	Proguanylin
ProUGN	Prouroguanylin
ROC	Receiver operating characteristic
RT-PCR	Reverse transcription polymerase chain reaction
STa	Heat stable enterotoxin
UGN	Uroguanylin

Chapter 1

Literature review

1.1 Heart failure

1.1.1 Introduction

THERE is no universally agreed definition of heart failure (HF), reflecting the complex pathophysiology of a condition that can arise from multiple different causes and lead to a highly variable clinical expression [1]. HF is classically described as a clinical syndrome characterised by symptoms of breathlessness and fatigue associated with signs of fluid retention, occurring as a result from any structural or functional cardiac disorder that impairs the ability of the heart to fill with or eject blood [2]. However Tan *et al* has criticized this description as being based on signs and symptoms which occur as a consequence of HF and suggested a more precise definition by first describing the heart's normal function in relation to the fundamental principles of thermodynamics and motion, illustrated in Figure 1.1. HF is thus defined as the inability of the heart under physiological conditions to convert chemical energy into adequate hydraulic energy at rates sufficient to maintain a circulation that copes with normal physiological stresses [3]. This definition avoids reference to specific physiological parameters such as cardiac output and ventricular function thus allowing a broader range of observed clinical phenomena to be classified as HF, as well as focussing attention on the underlying mechanisms of HF rather than clinical consequences.

Left ventricle (LV) systolic dysfunction (LVSD) is the most well studied form of cardiac pump impairment but in up to 50% of cases of HF, LV systolic function is preserved [4, 5]. In addition not all individuals with impaired LV function may have symptoms of breathlessness and fatigue or signs of fluid retention, indicating that LV dysfunction is necessary but not sufficient for the development of HF.

HF may develop rapidly as a result of acute myocardial injury to a previously healthy heart (*de novo* acute HF) or subacutely as a result of chronic myocardial damage. Once established, chronic HF is characterised by a gradual decline of myocardial performance associated with symptoms of breathlessness and fatigue interspersed with episodes of sudden acute deterioration (decompensated HF).

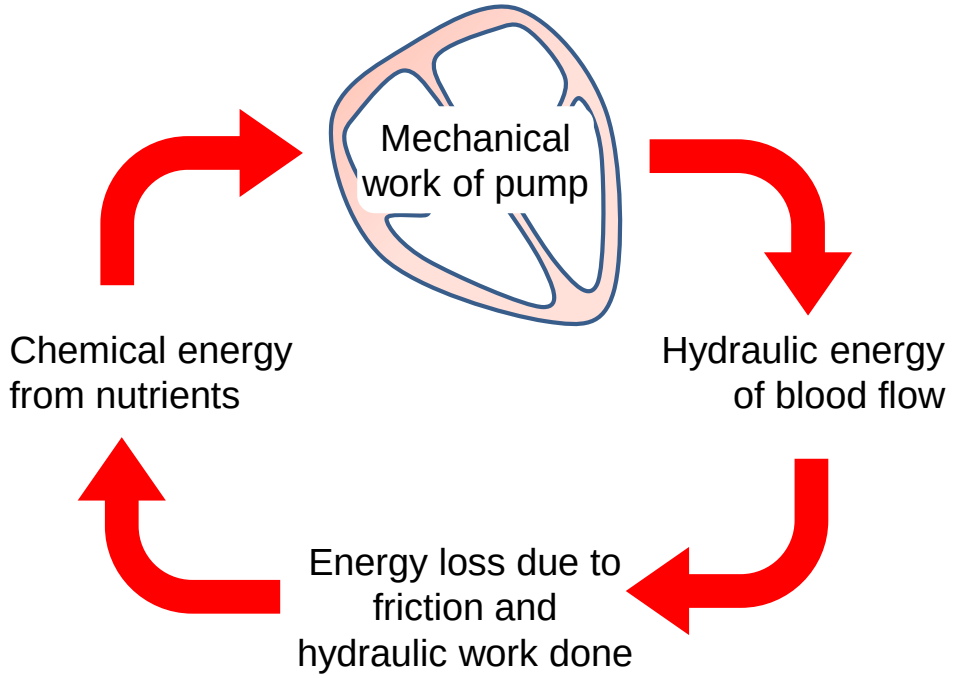

Figure 1.1 The function of the heart is to convert chemical energy supplied via nutrients into mechanical work which imparts hydraulic energy to the blood which is sufficient to maintain a physiological circulation. Hydraulic energy is the product of flow rate and pressure. Hydraulic energy is lost as heat due to friction with vessel walls and hydraulic work done, such as lifting the blood against gravity. The depleted hydraulic energy is then replenished by the heart pump [3].

1.1.2 Scope of the problem

Epidemiology

HF affects over 650,000 people in the UK [6] with approximately 27,000 new cases of HF diagnosed each year [7]. Data from the General Practitioner Research Database indicates that approximately 0.9% of men and 0.7% of all women have HF, with the highest prevalence in those aged over 75 years in whom it affects 13.7% of men and 12.5% of women [7].

Social and economic burden

HF inflicts chronic disability which imposes a large social and economic burden to individuals, their families and carers as well as wider society through social and health services. HF is estimated to cost primary care £45 million per year in consultations and £35 million per year in referrals to hospital outpatient clinics, as well as £129 million per year in community services [8]. In secondary care HF is responsible for 1 million inpatient bed days (2% of the total) as well as 5% of emergency admissions, an amount which is expected to rise by 50% over the next 25 years due to an ageing population [8]. Treating HF accounts for approximately 2% of the total National Healthy Service (NHS) budget of which 70% is as a result of hospitalisation after which up to 1 in 4 patients are re-admitted within 3 months [9]. In addition to the cost of medical and social care, the personal impact on individuals, their families and carers cannot be quantified.

1.1.3 Aetiology

Ischaemic heart disease (IHD) is the most commonly identified cause of HF in the UK responsible for approximately 52% of all cases in those aged under 75 years, followed by idiopathic cardiomyopathy (13%), valve disease (10%), and hypertension (4%) [10]. Rarer causes include drug and toxin induced, infiltrative and endocrine conditions, infections and peripartum cardiomyopathy [11].

1.1.4 Diagnosis

HF is diagnosed clinically by assessing a combination of symptoms, clinical signs and investigations which vary in sensitivity and specificity. The most commonly performed investigations are the electrocardiogram (ECG), chest x-ray (CXR), measurement of plasma natriuretic peptide concentration and transthoracic echocardiogram (ECHO). The positive and negative predictive values (PPV and NPV) of these investigations for an individual patient depends on the prevalence of HF in the subpopulation to which they belong.

Clinical signs and symptoms

The most common symptoms of HF are fatigue, breathlessness at rest and on exertion, orthopnea (breathlessness when lying supine) and paroxysmal nocturnal dyspnoea (sudden onset breathlessness occurring at night), while the most common signs are peripheral oedema, lung crepitations, an elevated jugular venous pressure (JVP), a displaced cardiac apex and third heart sound [8]. Since the sensitivity and specificity of these clinical features varies considerably depending on disease severity and patient population, patient history and examination are insufficient for the diagnosis of HF by themselves [8].

ECG

HF is associated with ECG abnormalities such as atrial fibrillation (AF), previous myocardial infarction (MI), LV hypertrophy, bundle branch block and left axis deviation. A meta-analysis combining summary receiver operating characteristic (ROC) curves of ECG abnormalities for the diagnosis of HF showed an overall area under the curve of 0.84 (95% C.I. [0.33 to 1.00]) [12], meaning that ECGs are an inadequate screening tool for HF as on average 16% of the cases would still be missed.

CXR

A meta-analysis of CXR changes for the diagnosis of HF showed that fluid redistribution identified preload with a sensitivity of 65% (95% C.I.[55 to 75])

and specificity of 67% [53 to 79] while cardiomegaly identified a decreased ejection fraction with a sensitivity of 51% [43 to 60] and specificity of 79% [71 to 85] [13]. However the poor inter-observer reliability for identification of pulmonary oedema on CXRs limits their diagnostic use. CXRs may be useful in the diagnosis of other causes of dyspnoea and are still recommended as part of the diagnostic assessment of HF in the 2010 NICE guidelines [8].

ECHO

ECHO allows direct visualisation of the ventricles and is thus the gold standard for diagnosis of LVSD [8, 11]. Observational studies show a strong inverse relationship between LV ejection fraction (EF) and mortality as well as non-fatal outcomes of re-hospitalisation and MI for patients with an EF of less than 45% [14]. However not all patients with HF have a reduced EF [4, 5]. This phenomenon is more prevalent in those who are older, overweight, female and have diabetes, hypertension or renal impairment and is almost always associated with impaired diastolic relaxation [15, 16]. Due to the uncertainty of the precise nature of mechanical dysfunction, the terms HF with preserved EF (HFPEF) or HF with normal ejection fraction (HFNEF) are preferred to the term diastolic HF [17]. Novel ECHO derived parameters such as fractional shortening and tissue Doppler are currently being evaluated as potential diagnostic markers of HFNEF/HFPEF [11].

Natriuretic peptides

Plasma atrial (A-type) natriuretic peptide (ANP) and brain (B-type) natriuretic peptide (BNP) are both elevated in patients with HF and inversely correlated with EF [18, 19] as well as being significantly associated with lower rates of survival than those with normal or only mildly raised levels [20, 21]. Plasma BNP levels are a significant predictor of all cause mortality in patients with both symptomatic and asymptomatic LVSD in meta-analysis [22]. In addition to prognosis in chronic HF, BNP has been shown to be diagnostic for HF in patients presenting with acute dyspnoea [23, 24]. The N-terminal fragment of the BNP precursor peptide, NTproBNP [25] has also been shown to have

prognostic value in patients with chronic HF [26, 27] as well as in patients following MI [28]. A systematic review comparing the diagnostic accuracy of NTproBNP and BNP in HF found no significant difference between the two [29].

Diagnostic criteria

The first diagnostic criteria for HF were derived from analysis of the Framingham Study cohort in 1971 [30]. Major criteria are PND or orthopnea, an elevated JVP, lung crepitations, cardiomegaly, acute pulmonary oedema, a third heart sound, increased venous pressure more than 16 cm of water, circulation time less than 25 seconds and hepatojugular reflux. Minor criteria are ankle oedema, night cough, dyspnoea on exertion, hepatomegaly, pleural effusion, reduction in vital capacity by one third from maximum and tachycardia with a heart rate more than 120 beats per minute. Weight loss could be a major criterion if more than 4.5 kg occurred over 5 days as a result of inpatient therapy. The diagnosis of HF was made if an individual met a minimum of 2 major or 1 major and 2 minor criteria, with the minor criteria not attributable to any other condition. Current guidelines use algorithms including ECHO and measurement of plasma BNP or NTproBNP levels [8, 11].

1.1.5 Assessment of severity

The New York Heart Association (NYHA) classification is the most widely used measure of severity of HF symptoms [11]. Patients are assigned into one of 4 categories (I to IV), with a higher class indicating a greater degree of limitation of physical activity and symptoms. NHYA class has been shown to have independent prognostic value for mortality in HF patients [31] but has high inter-observer variability [32] and only poorly correlates with EF and plasma NTproBNP levels whether assessed by a physician or the patient themselves [33]. More objective methods of severity assessment such as a 6 minute walk test and maximal exercise testing with measurement of peak oxygen uptake have been developed but are not in widespread clinical use [2].

1.1.6 Prognosis

HF has a poor prognosis, with one survey finding survival of 81% at one month following diagnosis falling to 57% at 18 months [34] and another finding a 5 year survival rate of 53% at 5 years in those with HF and LVSD [35]. This is similar to colon cancer but worse than breast or prostate cancer [8]. Survival rates are similar in HFNEF/HFPEF compared to HF with a reduced EF [36, 37].

1.1.7 Chronic HF

Understanding of the pathophysiology of HF has evolved from being considered a purely circulatory phenomena of reduced cardiac output due to impaired contractility to a complex disorder involving abnormal energy metabolism, cytokine activation, chronic inflammation, altered gene expression and disordered fluid balance [1]. Key to this conceptual shift was the discovery of ventricular remodelling as a response to myocardial damage, characterized by changes to heart structure and function extending from the intracellular level including expression of fetal β myosin chain isoforms, altered energy metabolism and defective calcium cycling in myocytes, to the extracellular level where there is fibrosis and inflammation [1]. These processes alter the extracellular matrix and electrical coupling between cells eventually leading to macroscopic changes in ventricular size and shape which progressively impair myocardial contractility [38]. The pathophysiological changes of HF are not only confined to the heart but induce systemic vascular, renal and endocrine compensatory responses which further depress cardiac function and accelerate the remodelling process. The interactions between these processes are summarised in Figure 1.2. The role of two aspects of systemic dysfunction in HF, neuroendocrine activation and natriuretic peptides are examined in the following sections.

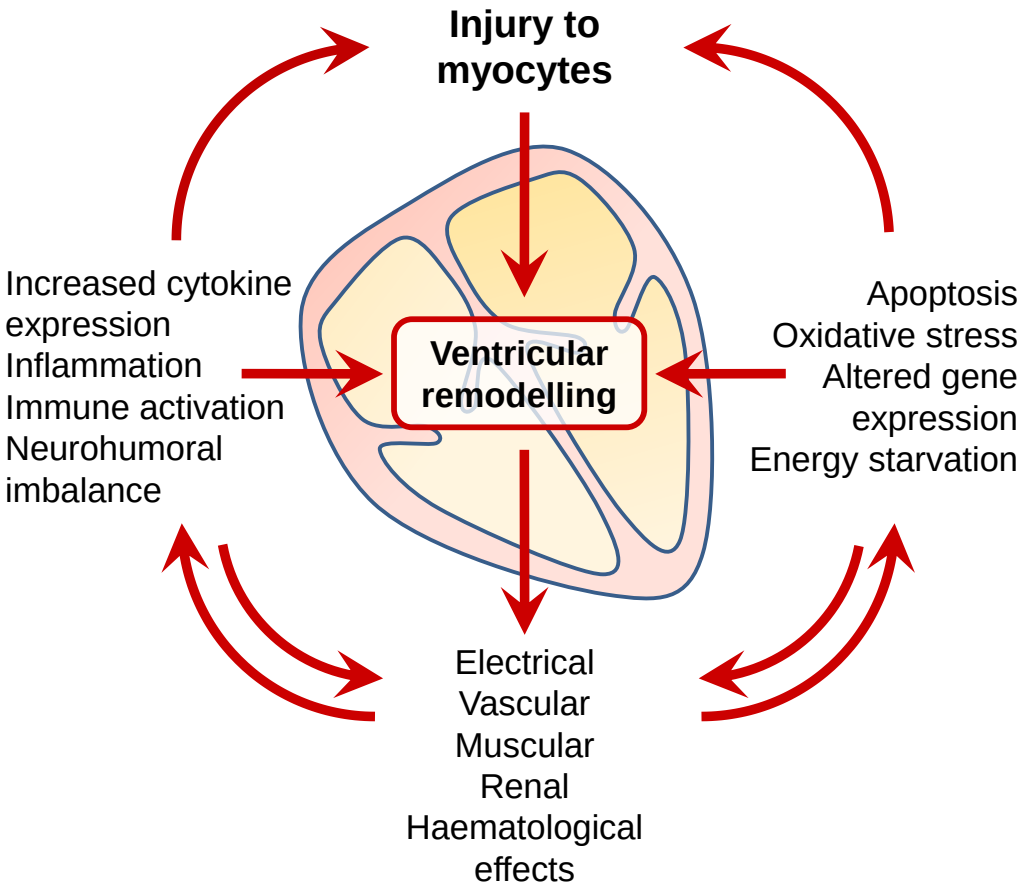

Figure 1.2 Summary of current understanding of pathophysiological mechanisms of HF. Myocardial damage triggers a chronic maladaptive response (remodelling) which alters the size, shape, and function the ventricles and in turn leads to progressive extra-cardiac dysfunction affecting the vascular, haematological, renal and endocrine systems, giving rise to the systemic manifestations of HF. Adapted from [39].

Neuroendocrine activation

The first evidence that HF involved a systemic disturbance in endocrine function came from the observation that venous hypertension or tissue anoxia alone did not reproduce the clinical picture of HF, demonstrating that purely mechanical pump failure could not lead to oedema formation in HF [40]. Early trials of salt and water restriction as well as the administration of mercurial diuretics improved patient's symptoms while increased sodium intake made oedema

worse, supporting the idea that salt played an important role in HF [41]. In particular it was observed that sodium and water excretion by the kidneys was reduced in HF patients although the mechanism of this was unknown [42]. These observations led to the hypothesis that sodium retention was essential for the formation of oedema [43], with an oral sodium tolerance test being proposed as a diagnostic test for HF [44].

The major mediator of renal sodium retention, the steroid hormone aldosterone, was first isolated in 1952 by Simpson and Tait [45] and has subsequently been found to be part of a complex neuroendocrine regulatory system involving the sympathetic nervous system and adrenal glands mediated by renin and angiotensin II which link the cardiovascular system and kidneys in the regulation of short and long term blood pressure and sodium balance [46, 47]. In addition to the regulation of sodium balance aldosterone has been found to be an important mediator of fibrosis and inflammation in cardiac remodelling, which contributes to progressive cardiac damage [48]. It has been suggested from an evolutionary perspective that activation of the neuroendocrine system is an advantageous response for maintaining cardiac output following acute haemorrhage but appears to be maladaptive when sustained as it leads to a cycle of progressive myocardial damage and falling cardiac output, illustrated in Figure 1.3 [49].

Patients with primary hyperaldosteronism (Conn's syndrome) however do not develop oedema, indicating that excess aldosterone alone cannot account for HF [50]. Although the mechanisms of sodium escape in Conn's syndrome are not known, natriuretic peptides which promote renal sodium excretion may play a potential role [51].

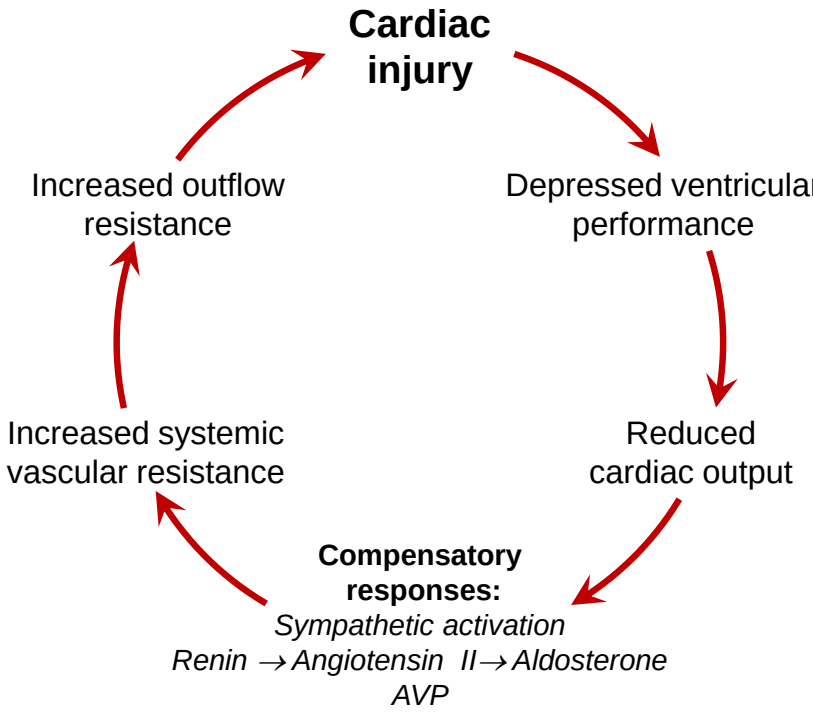

Figure 1.3 Reduced cardiac output stimulates chronic activation of neuroendocrine system in HF leading to a cycle of progressive myocardial damage and falling cardiac output. The main drug treatments for HF, betablockers, angiotensin converting enzyme inhibitors (ACEi) and aldosterone receptor antagonists (ARA) interfere with mediators of this cycle. Adapted from [46].

Natriuretic peptides

ANP and BNP promote renal sodium excretion by binding to a membrane bound guanylate cyclase (GC) receptor in renal collecting ducts, NPR-A [52–54]. Plasma ANP and BNP levels are elevated in patients with both chronic and acute HF and are mainly secreted from the atria and ventricles in response to increased wall stress, being correlated with severity of LV impairment and cardiac size [18, 19, 21]. Despite plasma ANP and BNP being elevated in patients with HF, sodium excretion remains impaired. There are several proposed mechanisms for this including defective circulating peptides [55, 56], increased expression of clearance receptors and neutral endopeptidases [57, 58], reduced renal NPR-A receptor expression and function [59] and increased intracellular cGMP degradation by phosphodiesterases [60]. A further important process may

be the effect of impaired renal perfusion and filtration of natriuretic peptides leading to reduced sodium and peptide delivery to the distal tubules resulting in impaired efficacy at their site of action [61].

Thus overall sodium balance in HF appears to be disturbed as a combination of chronic activation of the neuroendocrine system while at the same time there is reduced function of natriuretic peptides acting on the renal tubules, leaving patients with HF with a propensity for sodium and water retention. A proposed model for the failure of sodium homeostasis in HF is illustrated in Figure 1.4.

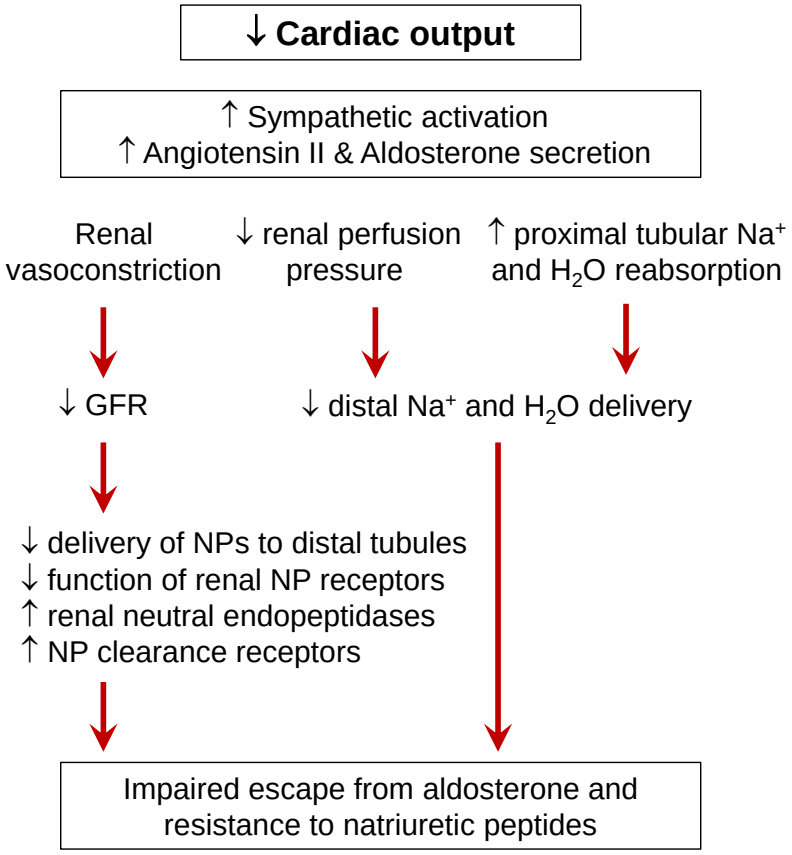

Figure 1.4 Failure of sodium homeostasis in HF. Impaired cardiac function leads to dysfunction of neuroendocrine and natriuretic peptide (NP) regulation of sodium and water balance predisposing to sodium and water accumulation. HF is stable (compensated) when sodium absorption and excretion are balanced, while episodes of decompensation are associated with excess sodium and water retention. Adapted from [62].

1.1.8 Acute HF

As acute HF may present with a range of clinical signs with a variable speed of onset depending on the cause and severity of previous myocardial damage, it is better understood as part of a spectrum of disorders named acute HF syndromes (AHFS). This is defined as a gradual or rapid deterioration in HF signs and symptoms requiring a need for urgent therapy [63]. Patients can be broadly divided into those with a gradual increase in peripheral oedema over days, those with hypertension and acute pulmonary oedema developing over hours and those presenting with hypotension and acute cardiogenic shock [63]. The high degree of variability in acute HF phenotype indicates that different mechanisms contribute in differing proportions in individual patients. The most common precipitant of *de novo* acute HF is acute coronary syndrome (ACS), with other causes including uncontrolled hypertension, peri-myocarditis and toxic injury being less frequent [64]. Deterioration in patients with chronic HF (decompensated HF) is associated with acute myocardial injury, dysrhythmias, intercurrent infection, renal injury, inflammation, endothelial dysfunction, acute neuroendocrine disturbance and non adherence to HF treatment [64]. Inflammation of the vascular endothelium [65] and sympathetic nervous mediated venoconstriction increasing preload and causing fluid redistribution [66] may also be important mechanisms for triggering venous congestion in those with chronic HF.

Weight gain associated with sodium and fluid retention is a predictor of hospital admission indicating that reduced renal sodium excretion may trigger decompensation in some patients [67]. Serial measurement of NTproBNP and BNP levels in patients with chronic HF show a considerable degree of natural variability [68] but the role of variation in plasma natriuretic peptide level and activity prior to episodes of decompensation remains unclear.

1.2 Guanylin peptides

1.2.1 An intestinal-renal natriuretic axis

Lennane *et al* investigated post-prandial response to oral sodium loads in rabbits [69] and human subjects [70] by measuring urinary sodium excretion following oral salt intake compared to after the same amount given intravenously. In both rabbits and in healthy human volunteers, urinary sodium excretion was significantly greater following oral sodium intake compared to after the same amount given intravenously, despite an overall sodium deficit by previous dietary salt restriction. This led Lennane to hypothesise the existence of an intestinal salt sensor mechanism which regulates sodium excretion in response to changing dietary intake. A number of mediators and mechanisms have been proposed to explain this phenomenon, including a hormone released by the liver, a hepato-renal nerve reflex and changes in plasma aldosterone levels.

Aldosterone suppression

Suppression of aldosterone release does not appear to mediate post prandial sodium excretion as plasma levels did not differ significantly following oral or intravenous salt load in rabbits [71] or healthy human subjects [72]. A possible hepatic hormone was investigated by comparing urinary sodium excretion after infusion of saline into the vena cava and hepatic portal vein in live rats [73]. Urinary sodium excretion was greater after the hepatic portal vein infusion, with no change in glomerular filtration rate (GFR), indicating that the liver may release a humoral factor into the circulation following dietary sodium load. This factor is yet to be identified.

A hepato-renal reflex

Evidence for a hepato-renal nerve reflex was studied by monitoring renal sympathetic nerve activity following infusions of saline into the hepatic portal vein. Increasing plasma osmolality in the hepatic portal vein of vagotomized rabbits by saline infusion induces a reflex decrease in renal sympathetic nerve activity,

suggesting the presence of hepatic sodium or osmoreceptors [74, 75]. This response was abolished by section of the hepatic nerves. Comparison of urinary sodium levels in monkeys before and after renal denervation showed there was significantly reduced post-prandial urinary sodium excretion following renal denervation [76]. These studies appear to support the hypothesis of a hepato-renal nerve reflex. However the earlier studies [74, 75] showed renal sympathetic nerve activity decreased following a hepatic portal vein sodium load, which would imply that no sympathetic nerve activity at all in denervated monkeys should elicit greater, not less post prandial natriuresis. Thus these observations cannot yet be adequately explained. Nonetheless, despite denervation, post-prandial natriuresis and diuresis still occurs indicating that additional factors are involved.

Role of ANP and BNP

Plasma ANP levels in healthy human subjects were significantly greater in those on a high sodium diet compared to a low sodium diet while plasma renin and aldosterone levels were significantly less [77, 78], with plasma ANP levels being positively correlated with urinary sodium excretion while being inversely related to plasma renin and aldosterone levels [79]. However oral water intake was not controlled in these experiments and so may have been a confounding factor.

The short term ANP response to a dietary sodium challenge was first reported in an informal study which found mean plasma ANP levels in healthy human volunteers increased after the consumption of salted potato chips [80]. However the quantity of sodium ingested and changes in water consumption and urinary sodium excretion were not measured, nor the timescale or dose response of plasma ANP levels. This observation was not supported by a more formal study which showed while urinary sodium excretion and osmolality increased after a high salt meal, plasma ANP levels did not change [81]. A study in healthy human volunteers further supported these observations, demonstrating that while plasma ANP levels briefly rose 15 minutes after a high salt meal, it decreased back to baseline levels before the increase in urinary sodium

excretion was detected [82]. However a post-prandial increase in urodilatin, a N-terminally extended form of ANP only synthesized in the kidney, was observed, which did significantly correlate with urine sodium excretion, indicating that this could be a potential mediator of post-prandial sodium excretion rather than ANP.

Comparison of plasma ANP levels in healthy human subjects on low, normal and high salt diets after oral versus intravenous sodium challenge showed a trend towards greater sodium excretion following oral salt intake compared to the intravenous route in all diets, although unlike earlier reports [70, 72] there was no significant difference in overall cumulative sodium excretion over 5 hours [83]. On all diets there was a significant 2 fold increase in plasma ANP compared to baseline following intravenous sodium challenge while only in the low salt diet group was there any elevation of plasma ANP levels after an oral salt load. The increase in ANP in response to oral salt load observed in the low salt diet group was delayed and smaller in magnitude compared to the rise in ANP following intravenous challenge. These findings suggest that ANP is not responsible for regulating renal sodium excretion in response to changes in dietary salt intake, except perhaps in those on a low salt diet.

BNP has not been investigated as a potential mediator of post-prandial sodium excretion. One study found significantly elevated BNP levels after five days of a high salt diet [84], but again water intake was not controlled so may be a confounding factor.

An intestinal natriuretic factor

Hansson *et al* investigated the potential production of natriuretic factors by the gastrointestinal tract by injecting purified fractions of homogenised intestinal tissue into cats and found a fraction containing material with a molecular mass between 500-1000 Da had a significant natriuretic effect [85]. This was the first experiment supporting the hypothesis of the existence of an intestinal natriuretic factor which is released in response to dietary salt intake and then travels via the circulation to the kidneys where it stimulates sodium excretion. Forte [86] hypothesised that members of the guanylin family of peptides were

the mediators of a putative intestinal-renal natriuretic axis. Guanylin peptides were originally discovered as endogenous ligands of the bacterial heat stable enterotoxin (STa) receptor, responsible for secretory diarrhoea due to pathogenic *E. coli*, one of the most common causes of infant mortality worldwide [87]. The discovery and characterisation of guanylins and their receptors is described in the following sections.

1.2.2 STa

STa was initially found to inhibit Cl^- absorption by rabbit ileal mucosal cells by the stimulation of GC leading to elevated intracellular levels of cyclic guanosine monophosphate (cGMP) [88]. STa shows competitive binding with radiolabeled ^{125}I-STa on T84 human colonic cells *in vitro*, indicating the presence of a specific STa receptor [89]. This led to the use of the T84 cells, which have morphological characteristics of differentiated colonic epitheilium, as a model for studying STa binding and stimulation of cGMP production as well as a bioassay for other potential STa receptor agonists. STa binding has been shown to occur on the brush border but not basolateral cell membrane of rat intestine enterocytes [90] as well as the small and large intestine, testes and proximal tubules of the kidneys in opossums [91]. STa binding is maximal at the villus tips, declining towards the crypts [92]. A similar cGMP response was seen to STa in OK cells derived from opossum kidney cortex [93]. As STa derived from intestinal bacterial is unlikely to enter the kidney tubules, this implied the existence of an endogenous STa receptor agonist. This hypothesis was supported by the observation that STa increased cGMP levels in opossum kidney cortex, medulla and intestinal mucosa tissue extracts *in vitro* as well as increasing urinary cGMP levels following intravenous injection into opossums [94]. The renal effects of STa were further demonstrated in isolated perfused rat kidneys where STa caused a marked increase in urinary excretion of both Na^+ and K^+ [95]. The accumulated evidence suggested that STa acted via a specific GC linked receptor for an as yet unidentified endogenous ligand.

1.2.3 The GC-C receptor

The STa receptor was discovered by searching an intestinal cDNA library using oligonucleotide primers based on conserved portions of GC, finding a novel GC receptor, named GC-C [96] as it was the third GC linked receptor to be found after GC-A (NPR-A) [52] and GC-B (NPR-B) [97] which are receptors for ANP, BNP and CNP. The extracellular portion of GC-C contains eight potential N-linked glycosylation sites and nine cysteine residues structurally divergent from those of GC-A and GC-B, thus making it specific for STa and failing to bind ANP and BNP [96]. However the intracellular domain contains both the protein kinase-like and GC catalytic domains, similar to those in GC-A and GC-B [96].

COS-7 cells transfected with GC-C mRNA showed STa induced cGMP accumulation as well as competitive binding with ^{125}I-STa, indicating that STa is a GC-C receptor agonist. Using northern blot analysis GC-C receptors were initially localized to rat small intestine only, with no expression in adrenal gland, brain, kidney, liver or lung tissue [96]. As northern blotting is a relatively insensitive method of detecting mRNA expression in tissues, the more precise method of semi-quantative reverse transcription polymerase chain reaction (RT-PCR) was subsequently used to identify sites of possible GC-C receptor expression. GC-C mRNA expression was found highest in the cortical collecting tubule, followed by the proximal convoluted tubule, medullary thick ascending limb and collecting tubule, and thin limbs of the loop of Henle in the rat nephron [98]. However these results were not replicated in a later study which found negligible GC-C receptor expression in rat kidney also using RT-PCR [99]. This may be partially explained by the earlier observations which found a high salt diet upregulates GC-C receptor expression in rat kidney, demonstrating that alterations in dietary salt intake can influence renal GC-C receptor expression [100].

GC-C receptor function has also been shown to be modulated by phosphorylation [101]. Addition of purified bovine protein kinase C (PKC) to T84 cells not only enhanced GC activity but also increased the number of ^{125}I-STa binding sites, although the mechanism of how this occurs is not known. This suggests that PKC either directly or indirectly via another kinase regulates GC-C receptor function by phosphorylation.

Although STa is a GC-C receptor agonist, STa may bind to other GC and possibly non GC linked receptors as is the case with ANP, BNP and CNP binding to GC-A, GC-B and the non GC linked clearance receptor NPR-C. ^{125}I-STa still binds to intestinal mucosa in GC-C$^{-/-}$ knockout mice, although at a lower level and not stimulating intestinal fluid secretion [102]. The nature of these receptors remains to be determined.

1.2.4 Guanylin

The discovery of a GC receptor specific for STa led to the search for an endogenous ligand. Purified fractions of rat jejunum tissue were tested for cGMP stimulating activity using a T84 cell culture bioassay, leading to the identification of a 15 amino acid peptide which increased intracellular cGMP levels and displaced radiolabeled STa from T84 cells [103]. This was named guanylin (GN) in reference to its activity on a GC linked receptor. Analysis of the amino acid sequence of GN revealed a high degree of homology to STa indicating that it likely stimulated the GC-C receptor via the same receptor binding region. GN mRNA is predominantly expressed in rat intestine and to a lesser extent in rat adrenal gland, kidney, oviduct and uterine tissue [104]. GN has been localized to goblet cells in both the small and large rat intestine in rats [105, 106] and enterochromaffin cells in guinea pigs using immunocytostaining [107].

The human homologue of GN was discovered using a rat cDNA probe to search a human cDNA library, isolating a 115 amino acid cDNA encoding a 15 amino acid peptide sharing 60-75% DNA and protein sequence similarity to rat GN [108]. This peptide stimulated cGMP production by T84 cells in the same way as rat GN and bacterial STa. GN expression in humans was initially found in the colon and ileum, and to a lesser degree the jejunum and ileum but not in the stomach using northern blot analysis [108]. GN mRNA was further localized to Paneth cells in bases of the crypts of the small intestine using in situ hybridization in humans [109].

Isolation of the human cDNA for GN shows that human GN is derived from a 115 amino acid precursor polypeptide [108]. Embyonic kidney cells transfected with a human GN cDNA encoding for a 115 amino acid peptide express a 94

amino acid precursor which is inactive until treated with trypsin [109]. Thus GN is synthesized as a propeptide, proguanylin (ProGN). Significantly higher levels of ProGN were found in the plasma of chronic HF patients compared to healthy controls using a radioimmunoassay [110].

GN induced Cl^- secretion when applied to the apical but not the basolateral side of murine intestinal mucosa when tested by measurement of short circuit current using an Ussing chamber [111]. The potential source of circulating GN or ProGN was investigated using an isolated vascularly perfused colon loop model which demonstrated that GN was released both intraluminally and into the portal effluent indicating that GN could potentially have an endocrine function [112]. Measurement of apical and basolateral GN and ProGN release from rat intestinal mucosa mounted in an Ussing chamber showed that under resting conditions, 15 times more ProGN than GN was released on the apical side while no ProGN and very small amounts of GN was released on the basolateral side [113]. Application of carbachol, a muscarinic receptor agonist, stimulated ProGN secretion 7 fold from both sides of the mucosa, indicating ProGN secretion may be partially under cholinergic control [113].

1.2.5 Uroguanylin

The observation that kidney tissue extract itself possessed cGMP stimulating activity on T84 cells [103] triggered the search for a renal source of GN. Two bioactive peptides which stimulated cGMP production in T84 cells were discovered in opossum urine using reverse phase high performance liquid chromatography (HPLC) and electrospray mass spectrometry [114]. The first was a 14 amino acid peptide 79% identical to human or rat GN likely to be the opossum homologue of GN while the second was a highly acidic 15 amino acid peptide sharing only 53% identity with GN. This was named uroguanylin (UGN) and was found to be 10 fold more potent than GN in stimulating cGMP production in T84 cells, but 10 times less so than STa. In addition it showed competitive agonism with ^{125}I-STa, binding to T84 cells with 10 fold more affinity than GN.

The human analogue of UGN was subsequently isolated from urine and characterised as a 16 amino acid peptide sharing sequence homology with

human GN as well as opossum UGN and GN, all having a conserved ACTGC COOH-terminal amino acid region [115]. However GN could not be detected in human urine, unlike opossum urine which contains a mixture of both GN and UGN [115]. Structurally UGN differs from GN by having two N-terminal acidic amino acids which are two Asp residues in human UGN and an Asp and Glu in opossum UGN. Human UGN further differs from GN by not having conserved aromatic amino acids which allow it to be hydrolysed by chymotrypsin to which UGN is resistant [116].

UGN mRNA expression in opossum tissue is highest in the duodenum as well as large intestine, atria and ventricles of the heart but not found in the kidney, liver or stomach using northern blot analysis [117]. Enterochromaffin and enterochromaffin like cells have been identified as the main cellular source of UGN in the rat gastrointestinal tract using northern blotting, immunocytochemistry and in situ hybridization, with expression highest in the duodenum, low in the stomach and distal small intestine and almost undetectable in the large intestine [118, 119]. As well as gut expression, UGN mRNA has also been localized in the rat nephron in a similar pattern to GN, except for the proximal convoluted tubule where expression of GN mRNA was high while UGN expression was almost undetectable [98]. A further study in rats found UGN mRNA expression was higher than GN mRNA with the highest levels in the proximal tubules while GN was mainly expressed in the collecting ducts [120].

Identification and sequencing of human cDNA for UGN revealed it codes for a 112 amino acid precursor, with expression initially found only in the colon [121]. A 24 amino acid N terminally extended bioactive form of UGN was found in human plasma and localized to entero-endocrine cells [122]. The full propeptide, prouroguanylin (ProUGN), was first isolated from opossum colonic mucosa and plasma [117, 123]. Opossum colonic mucosa contains a mixture or ProGN and ProUGN which are both unable to stimulate cGMP production by T84 cells until activation by V8 protease for ProGN and chymotrypsin for ProUGN [123]. While chymotrypsin activates ProUGN it inactivates ProGN by cleaving the GN peptide domain within it [123]. This was confirmed by cloning and expression of opossum ProUGN cDNA in a COS-1 cell line, which synthesized inactive ProUGN which was only activated with chymotrypsin [117].

ProUGN is the predominant form in human plasma while the 16 amino acid UGN was predominantly found in urine [124]. ProUGN is the main form in the intestine and kidney, localized to endocrine cells in the intestine and stomach, B cells in the pancreatic islets and tubular epithelial cells in the kidney [125].

The amino acid sequences of human and animal GN, UGN, *E. Coli* STa and lymphoguanylin, a related peptide sharing 40% homology with GN and 80% homology with UGN are shown in Figure 1.5.

Guanylin

Human	P G **T** C **E** **I** C A Y A **A** **C** **T** **G** **C**
Opossum	S H **T** C **E** **I** C A F A **A** **C** **A** **G** **C**
Rat/mouse	P N **T** C **E** **I** C A Y A **A** **C** **T** **G** **C**
Porcine/Guinea pig	P S **T** C **E** **I** C A Y A **A** **C** **A** **G** **C**

Uroguanylin

Human	N D **D** C **E** **L** C V N V **A** **C** **T** **G** **C** L
Opossum	Q E **D** C **E** **L** C I N V **A** **C** **T** **G** **C**
Rat/mouse	T D **E** C **E** **L** C I N V **A** **C** **T** **G** **C**
Guinea pig	N D **E** C **E** **L** C V N I **A** **C** **T** **G** **C**
Porcine	G D **D** C **E** **L** C V N V **A** **C** **T** **G** **C** S

Lymphoguanylin

Opossum	Q E **E** C **E** **L** C I N M **A** **C** **T** **G** Y

ST peptide

E. Coli	N S S N Y **C** C **E** **L** C C N P **A** **C** **T** **G** **C** Y

Figure 1.5 Comparison of the amino acid sequences of human and non-human GN-like peptides. GN has a Tyr-Ala (Y-A) bond is susceptible to chymotrypsin degradation while UGN has an Asn-Val (N-V) and *E. Coli* a Asn-Pro (N-P) bond that are resistant to chymotrypsin degradation (highlighted by boxes). Both GN and UGN have 4 Cys (C) residues enabling the formation of 2 intra-molecular disulphide bonds. Bold letters indicate conserved amino acids. Adapted from [126].

1.2.6 Pharmacological activity

The structural differences between GN and UGN cause important differences in pharmacological activity. UGN is more closely structurally related to STa than GN due to a conserved Asn^{109} residue, while human, rat and mouse GN peptides have an aromatic residue, Tyr or Phe in the same position [114] (see

Figures 1.5 and 1.6). As aromatic residues are cleavage sites for chymotrypsin, GN is sensitive to proteolytic cleavage and inactivation by this enzyme which is widespread in the gastrointestinal tract. Comparison of bacterial STa and GN receptor binding capacity in a suckling mouse model showed GN binding was reduced in the presence of chymotrypsin while STa was unaffected, indicating that GN is vulnerable to proteolytic cleavage while STa is not [127]. Substitution of the Tyr^{109}-Ala residues in GN with Asn^{109}-Pro conferred resistance to proteolytic inactivation as shown by the increased receptor binding of this modified peptide compared to unaltered GN in the presence of chymotrypsin [127]. As mentioned previously, the propeptide precursors of GN and UGN, ProGN and ProUGN also show differential modulation by proteases, with ProGN activated by V8 protease while ProUGN was activated by chymotrypsin, which in contrast inactivated ProGN [123].

The difference in chymotrypsin sensitivity between UGN and GN was demonstrated using the T84 bioassay, which showed cGMP responses to GN were reduced by $> 98\%$ in the presence of chymotrypsin while synthetic STa and UGN were unaffected [116]. The activity of GN and UGN was further modulated by pH, with UGN stimulating greater cGMP accumulation in T84 cells at pH 5.5 than at pH 8, while GN which stimulated greater intracellular cGMP accumulation when bioassayed at pH 8 than at pH 5.5.

The intestinal *in vivo* biological affects of GN peptides was assessed by intra-gastric injections of either STa, synthetic GN or synthetic UGN to suckling mice [128]. GN did not stimulate intestinal fluid secretion while UGN and STa did, STa being a more potent stimulant than UGN. Co-administration of chymostatin and aprotinin, protease inhibitors, with GN significantly increased intestinal fluid secretion almost to the same amount as STa. This indicates that *in vivo* the activity of GN is markedly reduced by the presence of intestinal chymotrypsin, which is secreted by the pancreas, while UGN and STa are not. The relative potencies of GN, UGN and STa were compared by measuring cGMP production in isolated loops of mouse intestine *in vitro*, which showed in the absence of proteases, STa was the most potent while GN and UGN had similar potency, showing that the difference in observed *in vivo* effects of GN and UGN were likely due to differential inactivation by proteases rather than intrinsic

differences in potency.

The biological significance of differential modulation of UGN and GN activity by proteases and pH within the intestine remain unclear. The pH of the intestinal microenvironment ranges from less than 6.0 to greater than 8.0 for the fluid bathing enterocytes while the lumen content pH varies from less than 3.0 to greater than 8.0 [116]. In addition STa has been shown to induce surface alkalinisation of small intestine mucosal surface by stimulation of GC-C receptors in mice [129]. Thus GN and UGN are likely to not only exert different degrees of paracrine and autocrine activity on enterocytes at different sites along the gastrointestinal tract depending on the local pH, but also may actively modulate mucosal surface pH. This may serve a protective function, alkalinising the mucosal surface in response to acid gastric chyme output into the jejunum.

The influence of pH on GN and UGN activity was further investigated *in vitro* by measuring cGMP accumulation after peptide challenge in T84 cell assays with different growth medium pH [130]. GN was 10 times more potent at pH 8.0 compared to at pH 5.0, with UGN showing the opposite response. At pH 5.0 UGN was 100 times more potent than GN, while at pH 8.0 GN was 3 times more potent than UGN. Similar responses were observed when Cl^- secretion was measured in different pH environments. pH induced differences in potency are accounted for by changes in binding affinity for the GC-C receptor, with GN showing 100 times greater affinity at pH 8.0 compared to pH 5.0, and UGN showing 9 times greater affinity at pH 5.0 compared to pH 8.0. Removal of the N-terminal Gln^{95}, Glu^{96} and Asp^{97} from opossum UGN increased its potency at high pH, showing that these acidic residues are responsible for the increased binding affinity and potency of UGN at low pH.

Modulation of activity of UGN and GN by pH may also have physiological consequences in the nephron. The intraluminal environment becomes progressively more acidic distal to the proximal tubule, favouring the activity of UGN rather than GN [131].

1.2.7 Stereoisomers

The presence of two or more cysteine residues in a polypeptide chain allow the formation of disulfide bridges which fold the chain into non planar conformations, forming topological stereoisomers which may have distinct biological properties [132]. GN, with four cysteine residues and two disulfide bridges has been shown *in vitro* to have two distinct topological stereoisomers using nuclear magnetic resonance (NMR) spectroscopy [133] although this has not yet been shown to occur *in vivo*. When synthesized artificially only the GN isomer closest in structural similarity to STa showed biological activity, stimulating a short circuit current in murine mucosa mounted in an Ussing chamber, while the other isomer showed no response and did not antagonise the active isomer [111]. UGN, also having four cysteine residues, has two stereoisomers, with one stereoisomer having only 10% of the maximal cGMP stimulating activity of the other in the T84 cell bioassay [134]. Plasma concentrations of both stereoisomers of UGN have been measured using a radioimmunoassay, finding that the bioactive form was present at a higher level in the plasma (5.0 ± 0.3 fmol·ml^{-1} [mean ± SE]) than the inactive form, (1.6 ± 0.1 fmol·ml^{-1}) [124]. Both isoforms of UGN can interconvert depending on the pH and temperature of the solution [135]. This could lead to variability in detection if the T84 bioassay is used, as one isomer is much less potent than the other at stimulating cGMP production. The amino acid sequences of human GN and UGN showing the location of the disulfide bridges are shown in Figure 1.6.

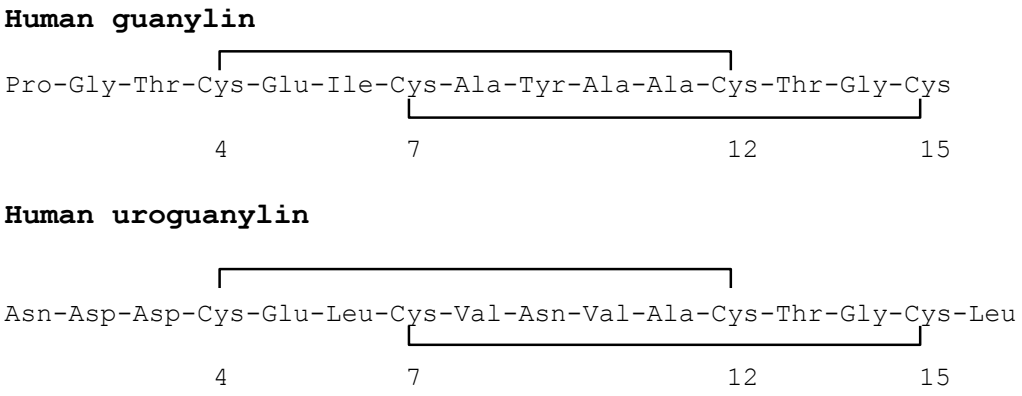

Figure 1.6 Comparison of amino acid sequences of human GN and UGN, showing the positions of disulfide bonds as black lines. Adapted from [126].

1.2.8 Mucosal intracellular signalling

Early *in vitro* experiments on rabbit ileal mucosal cells established that STa inhibits Cl^- absorption and stimulates GC leading to a rise in intracellular cGMP but not cyclic adenosine monophosphate (cAMP), indicating that cGMP is involved in mediating STa induced Cl^- secretion [88]. Subsequent experiments sought to resolve whether GC-C receptor induced anion secretion was mediated by cGMP directly or indirectly via protein kinases such as cGMP dependent protein kinase (PKG) or cAMP dependent protein kinase (PKA) as well as the identity of the channel responsible for Cl^- secretion.

Comparison of intragastric injection of STa, 8-bromo cyclic GMP and 8-bromo cyclic AMP (membrane permeable analogues of cGMP and cAMP respectively) into mice showed that STa induced a rise in cGMP levels which preceded intestinal fluid secretion, while no rise in cAMP levels was observed [136]. Stimulation of intestinal fluid secretion by STa was almost exactly reproduced by administration of 8-bromo cyclic GMP and but also by 8-bromo cyclic AMP. This suggests that cGMP is the main mediator of intestinal fluid secretion in response to STa although increased intracellular cAMP activity could produce a similar effect, despite the fact that intracellular cAMP level itself was not elevated following stimulation of gastric secretion by STa. Apical application of

STa to T84 colon carcinoma cells *in vitro* induces Cl^- secretion with a parallel increase in intracellular cGMP but not cAMP or Ca^{2+} levels, again supporting the hypothesis that cGMP is the main mediator of Cl^- secretion [137].

Further experiments showed that not only did phosphorylation by PKG stimulate Cl^- channel opening but that the catalytic unit of PKA could also induce Cl^- secretion [138]. The potential role of PKA was supported by the observation that a PKA inhibitor suppressed Cl^- secretion more effectively than a PKG inhibitor in T84 cells [139]. Chromatographic analysis showed however that there were low levels of PKG in T84 cytosol whereas PKA was present at much higher concentrations [140]. This likely explains why cGMP cross-phosphorylation of PKA seems to occur in T84 cells. Type II but not Type I PKG was found to activate Cl^- secretion by the CFTR in IEC-CF7 cells from a rat intestinal cell line expressing recombinant CFTR [141]. Type II PKG was localized to the brush border of the apical membrane of the duodenum to proximal colon in the rat while the Type I isoenzyme was only present in the smooth muscle cells of the lamina propria [142]. T84 cells did not express either Type I or II PKG, thus again explaining earlier observations which found that cGMP stimulated Cl^- secretion in T84 by cross phosphorlylating PKA, as there is none or very little PKG present in this cell line. *In vivo* experiments in PKG Type $II^{-/-}$ mice showed increased fluid absorption under resting conditions compared to wild type in all intestinal segments, while a STa challenge failed to induce net fluid loss in the the ileum and jejunum, indicating that PKG Type II is an important *in vivo* mediator of STa provoked intestinal fluid loss [143].

The Cl^- channel involved was identified as the cystic fibrosis transmembrane conductance regulator (CFTR) by pretreating T84 cells with anti-sense DNA to CFTR, finding that STa induced Cl^- secretion was attenuated and CFTR expression reduced compared to controls pretreated with missense oligonucleotides [139]. Transfecting NIH-323 fibroblasts with CFTR induces a Cl^- current after stimulation by the cGMP membrane permeable analogue, CPT-cGMP, while untreated NIH-323 fibroblasts which do not naturally express CFTR showed no response [139]. The I_{sc} response to UGN was significantly reduced but not abolished in proximal duodenal tissue from $CFTR^{-/-}$ mice [144]. Although these experiments provide evidence that CFTR has a significant role

in GN and UGN induced Cl^- secretion, they also provide evidence of an additional anion secretion pathway. A potential candidate for this is the ClC-2 Cl^- channel which has been found in the duodenum of both wildtype and $CFTR^{-/-}$ mice [145].

As well as the effect of STa on Cl^- secretion, the effect on intestinal epithelial Na^+ transport was also studied using an Ussing chamber. Under basal conditions wild type jejunal epithelium showed overall net Na^+ absorption, but when STa was added this became net excretion, due to inhibition of Na^+ absorption. STa added to PKG Type $II^{-/-}$ jejunal epithelium also inhibited Na^+ absorption but only by approximately 50% compared to wild type epithelium [143]. Thus the GC-C receptor PKG Type II linked pathway appears to not only stimulate Cl^- secretion, but also inhibit intestinal epithelial electroneutral Na^+ absorption. Current understanding of the signalling pathway from GC-C receptors in intestinal mucosal cells is illustrated in Figure 1.7.

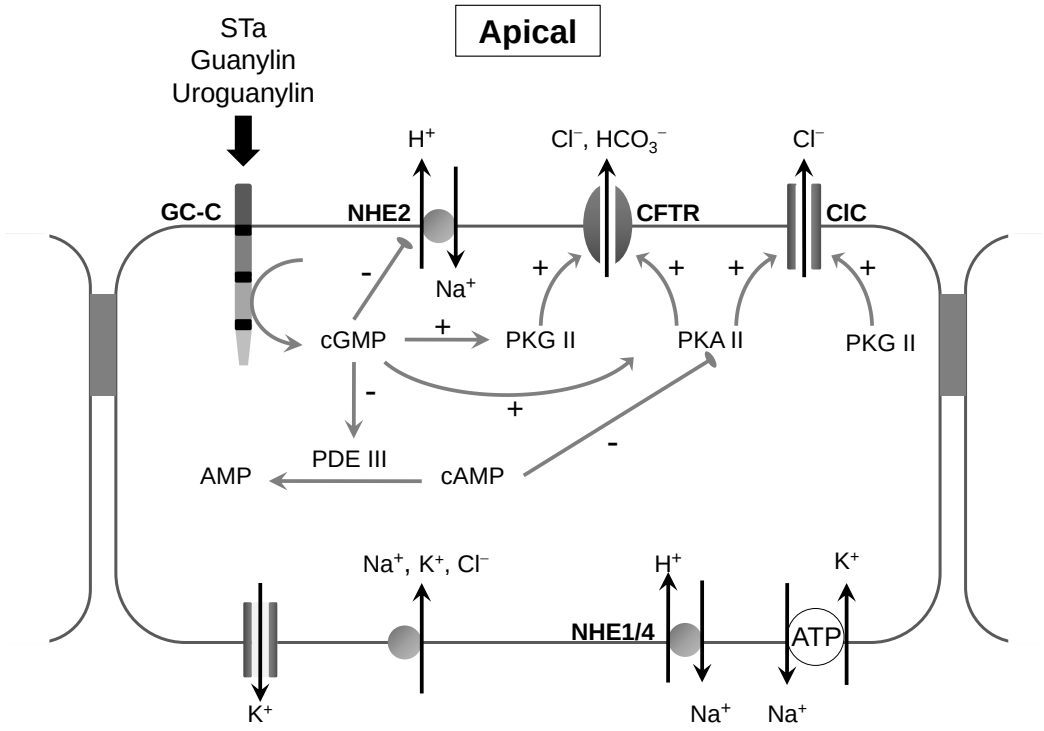

Figure 1.7 Proposed intracellular signalling pathways in intestinal mucosal cells. Elevated intracellular cGMP produced by activation of GC-C receptors stimulates increased Cl^- and HCO_3^- secretion from CFTR and ClC anion channels by three main mechanisms: 1)Phosphorylation of cGMP dependent protein kinase II (PKG II), 2)Phosphorylation of cAMP dependent protein kinase II (PKA II), 3) Inhibition of phosphodiesterase III (PDE III), elevating intracellular cAMP. Adapted from [126].

1.2.9 Renal intracellular signalling

Infusion of GN and UGN into isolated perfused rat kidneys induces natriuresis, kaliuresis and diuresis, with UGN being more potent than GN [146]. However GN and UGN also induce these effects in GC-$C^{-/-}$ mice, indicating that a GC-C receptor independent pathway is present in the renal tubular epithelial cells [147]. An *in vitro* study on a renal proximal tubule epithelial cell line, IHKE-1, showed that there are two distinct signalling mechanisms present, a GC-C receptor cGMP dependent pathway and a cGMP independent pathway stimulated by an unknown receptor [131]. Pertussis toxin inhibits hyperpolarisation of IHKE-1 cells by UGN but not GN, indicating that UGN also stimulates

a pertussis sensitive G protein linked receptor in proximal tubule cells. A proposed model for intracellular signalling in proximal renal tubule cells is shown in Figure 1.8.

GC-C receptor mRNA is not expressed in human cortical collecting duct (CCD) cells which undergo depolarization in response to GN and UGN likely as a result of inhibition of ROMK K^+ channels [148]. Inhibition of phospholipase A_2 (PLA_2) blocked CCD cell depolarization, indicating that G protein linked arachidonic acid mediated pathway may be responsible for natriuresis and diuresis in response to GN and UGN by reducing the driving force for Na^+ and water resorption.

In mouse CCD cells inhibition of PKG and basolateral K^+ channels also blocks hyperpolarization in response to GN and UGN, indicating the presence of a GC-C receptor independent PKG mediated pathway [149]. The most recent study by Qian *et al* [99] confirms that the GC-C receptor is not found in rat kidney although ProUGN is, supporting previous observations that the renal actions of guanylins are mediated via different receptors. A proposed model for intracellular signalling in CCD cells is shown in Figure 1.9.

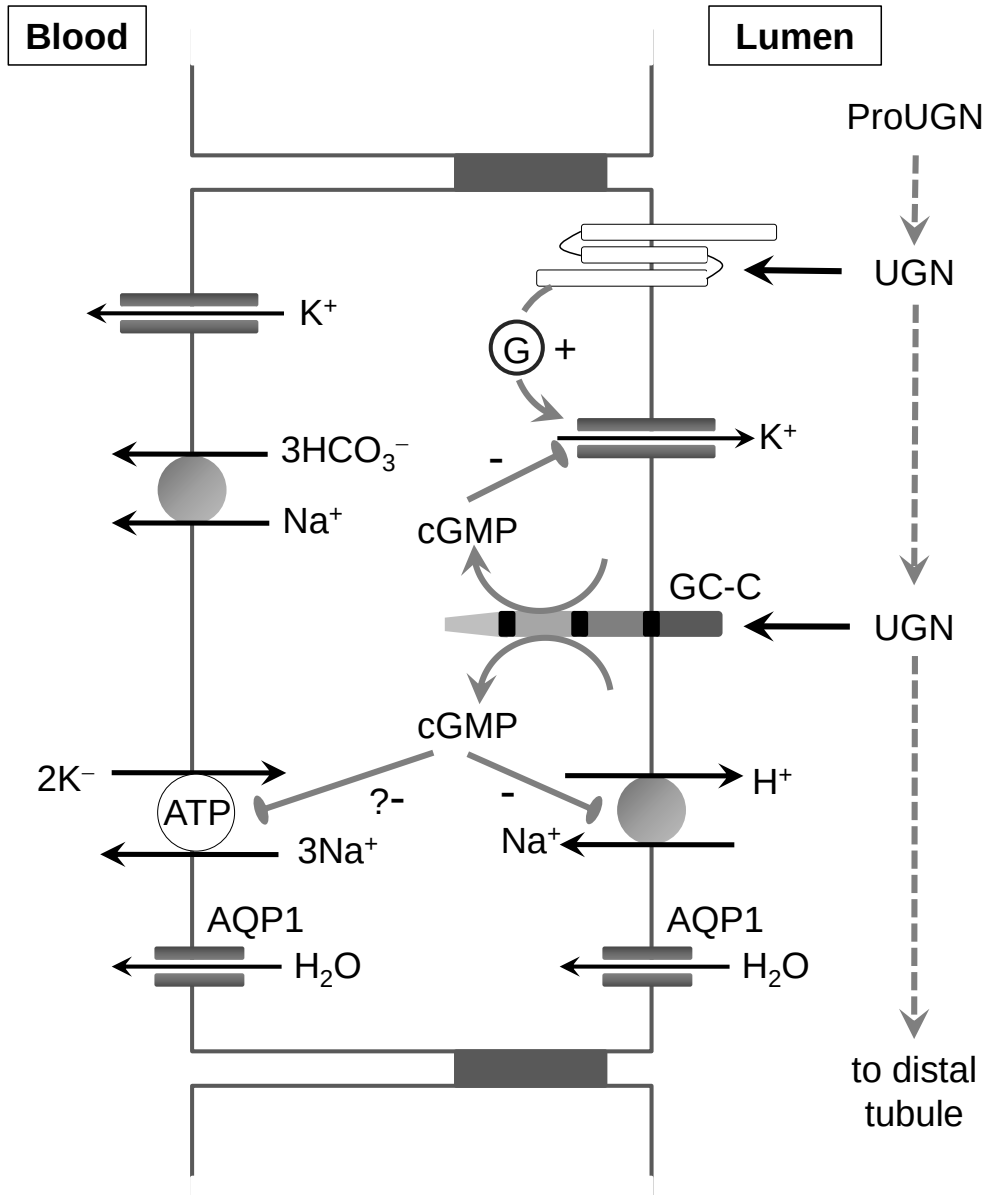

Figure 1.8 Proposed intracellular signalling in proximal renal tubule cells. GN and UGN induced natriuresis and kaliuresis likely occurs via two distinct mechanisms, one by a GC-C receptor cGMP linked pathway and the second via a pertussis sensitive G protein linked receptor. Activity of GN and UGN on the GC-C receptor pathway may be modulated by intraluminal pH as UGN has greater potency than GN in acidic conditions. UGN can also act on a GC-C receptor independent pathway which is blocked by pertussis toxin, likely via a pertussis sensitive G protein linked receptor. Adapted from [150].

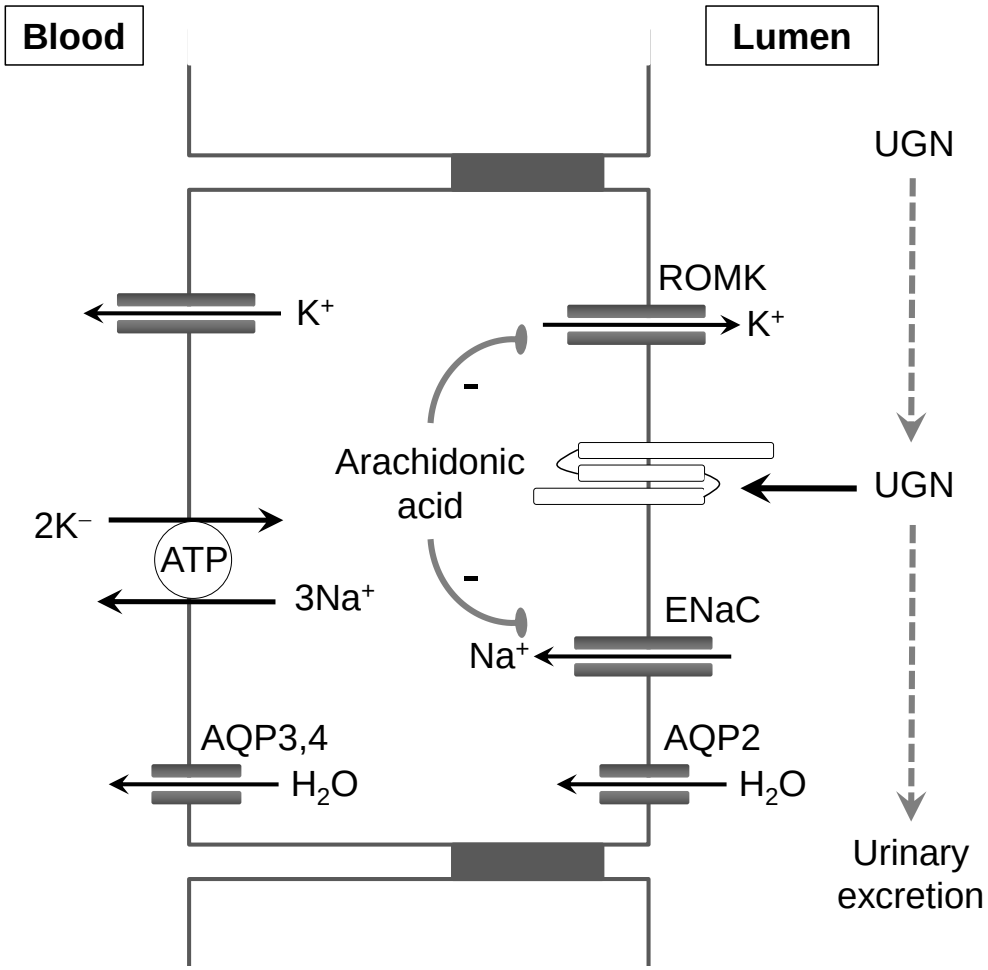

Figure 1.9 Proposed intracellular signalling in renal cortical collecting duct (CCD) cells. GN and UGN may induce natriuresis and kaliuresis by two possible mechanisms, one via a G protein linked PLA_2 receptor which stimulates arachidonic acid production which inhibits ROMK K^+ channels and the other via PKG and cGMP mediated activation of basolateral K^+ channels. The GC-G receptor which is present in mouse CCD cells may be the receptor activating this pathway [151]. Adapted from [152].

1.3 Physiology of guanylin peptides

1.3.1 Knockout models

$UGN^{-/-}$ mice have reduced urinary excretion of sodium in response to an enteric sodium load compared to wild type [153]. In addition they have a significantly greater mean arterial blood pressure than wild type mice regardless of dietary salt intake which suggests UGN may play a role in blood pressure regulation [153]. However these results may have been confounded as $UGN^{-/-}$ mice also had reduced levels of GN expression despite the GN gene being unaffected.

GC-$C^{-/-}$ receptor mice however show similar urinary sodium excretion in response to exogenous UGN or STa as do wildtype likely by reducing tubular sodium resorption [147]. Urine cGMP excretion in response to exogenous UGN is also unaffected in GC-C receptor knockout mice. This result demonstrates the presence of an non GC-C mediated receptor in the kidney. Elistur *et al* [154] further compared sodium excretion in GN, UGN and GC-$C^{-/-}$ receptor knockout mice in response to low and high salt diets. UGN knockout mice have significantly reduced sodium excretion in response to a high salt diet compared to wildtype while sodium excretion was unaffected in GN and GC-C receptor knockout mice. As observed by Lorenz [153] urinary cGMP excretion was generally lower in UGN knockout mice and did not increase in response to a high salt diet. Urinary cGMP excretion did not increase in response to a high salt diet in GN and GC-C receptor knockout mice either.

1.3.2 Role in sodium balance

If guanylin peptides are involved in the regulation of gut sodium absorption and renal sodium excretion then plasma levels should rise after oral salt intake followed by an increase in renal sodium excretion. Since guanylin peptides are produced both in the gut and the renal tissue itself, increased renal sodium excretion following oral salt intake could be due to increased renal synthesis of guanylins or as a result of increased gut derived peptide travelling to the kidneys via the circulation. The effects of oral salt intake on intestinal and renal

expression of guanylins, whether oral salt intake changes plasma peptide levels and the renal effects of increased plasma levels are described in the following sections.

Gut and renal expression

The effects of oral salt intake on intestinal guanylin expression has been investigated in rats fed on low, normal and high sodium diets for 1 week [155]. There was reduced colonic GN mRNA expression and ProGN production in rats on a low sodium diet compared to rats on a normal salt diet, while GN mRNA and ProGN peptide expression was not increased in the high salt diet group compared to normal. GC-C activity was reduced in the low salt group compared to the normal group, with there being no difference between the normal and high salt diet groups. These results indicate colon specific downregulation but not upregulation of GN and GC-C receptors in response to variation in dietary salt intake. Another group found rats fed a high salt diet for 4 days had increased expression of GN mRNA in duodenum and jejunum but not UGN [156]. Oral salt challenge to rats previously fed a low salt diet stimulated both GN and UGN mRNA expression throughout the gastrointestinal tract within 4 hours, while a sodium challenge to rats previously fed a normal salt diet stimulated only GN mRNA expression in the duodenum and jejunum and UGN mRNA expression only in the ileum and colon. This may explain an earlier observation that urine sodium excretion was only greater following oral salt challenge compared to intravenous challenge in sodium depleted rats but not in rats maintained on a normal diet [157].

The effects of oral salt intake on renal guanylin peptide expression were tested by feeding mice a 1% salt solution for 3 days [120]. Mouse renal UGN mRNA expression significantly increased 1.8 fold, but renal GN mRNA expression was not altered . Both UGN and GN renal mRNA expression were not affected by low salt diets, salt loading with food or dehydration.

Change in plasma levels

Measurement of plasma and urine UGN in rats after a one week low or high salt diet found that while urine UGN levels were significantly elevated in the high salt diet group compared to the low salt diet group, plasma levels were unchanged [158]. Renal and intestinal UGN mRNA expression were also increased in the high salt diet group. This result supports the previous studies showing elevated UGN production in response to high oral sodium intake and further demonstrated that UGN synthesis was upregulated in both the intestine and kidney. However this study looked at steady state plasma UGN levels achieved after a one week high sodium diet, not rapid short term changes in plasma ProUGN and UGN levels after an oral salt challenge. 24 h urinary UGN excretion in human subjects was significantly greater in those on a high salt diet compared to a low salt diet, with significant positive correlations between urine UGN excretion and urine Na^+, K^+, Cl^- and cGMP excretion [159]. Plasma ANP and BNP did not significantly correlate with urinary Na^+, K^+ and Cl^- excretion, but did correlate with urinary cGMP excretion. The post-prandial response in human subjects fed a standard meal showed a rise and fall in plasma ProUGN over 150 minutes, although the sodium content of the meal was not specified [160].

Renal effects

Intravenous injections of STa and UGN in mice significantly increased urine volume, sodium and potassium excretion compared to controls while GN only showed a trend towards these effects [128]. Infusion of UGN and GN into isolated rat kidneys was similarly found to induce natriuresis and kaliuresis, with UGN having the more potent effect [146]. The more potent time and dose dependent natriuretic and kaliuretic effect of UGN compared to GN following intravenous administration was again found by Carrithers *et al* [161] in an *in vivo* mouse model. There was no change in glomerular filtration rate, plasma creatinine, urine osmolality, blood pressure or heart rate following infusion of UGN implying that UGN acts by affecting renal tubule transport rather than altering cardiovascular haemodynamics. Pre-treatment of isolated perfused rate kidney with a trypsin and chymotrypsin inhibitor significantly increased

urine flow and sodium excretion in response to GN compared to without pre-treatment, when GN has no natriuretic effect [162], suggesting that the low levels of GN in mammalian urine [115] as well as the the low *in vivo* natriuretic potency of intravenous GN may be due to degradation by chymotrypsin like proteases in the renal tubules.

Evidence of endocrine activity

As intravenous injections of GN only have limited natriuretic effect, Moss and Qian *et al* hypothesised that UGN or ProUGN was the mediator of an intestinal-natriuretic axis and investigated this possibility in a detailed series of experiments. In rats, the ratio of plasma ProUGN to UGN was 40:1, thus plasma levels of UGN were less than 2.5% than that of ProUGN, establishing that ProUGN was the dominant form of the peptide in rat plasma [163]. Intestinal portal vein ProUGN levels were 177 ± 16% of arterial plasma, demonstrating that the intestine was likely a major source of circulating ProUGN. Removal of the intestine reduced plasma ProUGN levels to 27% of their baseline levels, while renal vein ProUGN levels were 63 ± 5% of renal artery levels showing that the kidney clears ProUGN from the circulation rather than contributing. Infusion of ProUGN at physiological levels stimulated urinary sodium excretion and urine flow demonstrating it could act in an endocrine fashion, most likely through mechanisms within the tubule and not by altering cardiovascular haemodynamics which were not affected. This study differed from previous measures of ProUGN as a western blot based immunoassay able to accurately distinguish between ProUGN and UGN was used unlike previous studies which used radioimmunoassays with antibodies against the C terminal sequence which could not.

Injections of ^{35}S radiolabeled ProUGN into rats showed that circulating ProUGN rapidly accumulated in the kidney where it is metabolised within the renal tubules with the majority of fragments excreted into the urine and not metabolised in the plasma compartment [164]. The elimination curve of injected ProUGN closely mimicked that of inulin, indicating that circulating ProUGN most likely enters the nephron by passive glomerular filtration rather

than active excretion by tubular epithelial cells [164]. In addition, injections of UGN had a greater antikaliuretic effect than ProUGN, especially at low plasma concentrations. Infusions of ProUGN however only result in very low amounts of UGN in the urine relative to the amount infused compared to infusions of UGN [165]. Thus it appears that while ProUGN undergoes metabolism in the renal tubules, it is not metabolised into UGN but into another peptide which exerts a predominantly natriuretic action while UGN has a predominantly antikaliuretic function.

Summary of role in sodium balance

The evidence described in the previous sections shows guanylins have a role in intestinal and renal regulation of sodium excretion. However the relative importance of guanylins compared to the neuroendocrine system and other natriuretic peptides ANP and BNP in regulation of short and long sodium balance in humans is yet to be determined. The evidence that guanylins mediate an intestinal-natriuretic axis is not yet conclusive, but indicates that ProUGN could be the most likely mediator of such a system. A time and dose response relationship between oral salt intake and changes in plasma ProUGN levels with corresponding increases in renal sodium excretion would need to be demonstrated to support this hypothesis.

The major role of guanylins may be in the local regulation of sodium absorption in the gut. Renal sodium excretion appears to be increased particularly after periods of salt restriction, which seems paradoxical. However rapid changes in plasma sodium can have serious neurological consequences, especially when plasma sodium concentrations are low [166]. Thus guanylins may serve a protective function acting as a "salt buffer", preventing rapid increases in plasma osmolality.

1.3.3 Other physiological roles

As well as the regulation of sodium balance, there is recent evidence guanylins and the GC-C receptor are involved in appetite regulation [160] and in the pathology of intestinal adenocarcinomas. Guanylin is downregulated mouse

and human intestinal adenomas [167] and loss of GC-C receptor expression is associated with increased risk of colon cancer in mice [168], while GC-C$^{-/-}$ and GN$^{-/-}$ mice have increased proliferation of colonic epithelial cells and abnormal villus crypt architecture [169]. Li *et al* have hypothesised that guanylin peptides acting on intestinal GC-C receptors may serve a paracrine tumour suppressor function, which when dysregulated predisposes to tumorigenesis [170].

1.3.4 Role in HF

UGN excretion has been found to be 70 times increased in the urine of HF patients compared to healthy controls [171]. This study is the first evidence to suggest that UGN synthesis may be upregulated in HF patients and may be an adaptive response to sodium retention in HF. However this study leaves many questions unanswered. Plasma UGN and plasma and urine ProUGN levels were not measured so it was not possible to determine whether the source of the increased UGN production in HF patients was from the intestine or from the kidney or whether the processing of ProUGN into UGN is altered in HF patients compared to healthy controls. Also the T84 bioassay was used to measure UGN levels, not an immunoassay, so may not accurately reflect UGN levels or distinguish between other GC-C receptor activating peptides. Furthermore the impact of HF severity on plasma and urine UGN levels as well as how renal dysfunction may alter ProUGN processing into UGN remains to be determined. A better understanding of the UGN system would enable potentially new diagnostic, prognostic and therapeutic advances in the management of HF.

1.4 Study aims and hypotheses

The aim of the study is to investigate the role of ProUGN and ProGN in chronic and acute (decompensated) HF associated with signs of fluid retention. The following hypotheses were tested:

1. Plasma ProUGN and ProGN levels are deranged in chronic HF compared to healthy controls.

2. Plasma ProUGN and ProGN levels are altered according to the severity of HF.
3. Plasma ProUGN levels change during treatment and recovery from admission with acute HF, with the magnitude of change corresponding to clinical outcomes of all cause mortality and readmission with HF.
4. Resistance to the physiological effects of ProUGN measured by ProUGN/cGMP ratio occurs in patients acute HF, and is associated with adverse outcomes of mortality and readmissions with HF.
5. Plasma ProUGN, cGMP and ProUGN/cGMP ratio contribute independent prognostic information on patients with decompensated HF, in terms of mortality and readmissions with HF.

Chapter 2

Methods

2.1 Subject recruitment

This study abided by the Declaration of Helsinki and was approved by the local ethics committee. Written informed consent was obtained from all subjects. The study comprised of 3 different cohorts:

1. Healthy controls
2. Patients with acute decompensated HF
3. Patients with chronic stable HF

2.1.1 Healthy controls

Normal controls were recruited from the University of Leicester. All controls took no regular medications, had no history of hypertension, diabetes, ischaemic heart disease and had no ECG or ECHO abnormalities.

2.1.2 Acute HF patients

Patients with acute HF were recruited from the admission units of the Glenfield Hospital and Leicester Royal Infirmary. Acute HF was diagnosed in patients fulfilling the following diagnostic criteria:

1. History of worsening or new onset shortness of breath

2. Compatible clinical signs of pulmonary rales or elevated JVP or peripheral oedema
3. HF as the primary diagnosis at admission

Patients could already have a diagnosis of chronic HF or be a first presentation of acute HF. Patients with a different primary diagnosis, for example acute coronary syndrome or respiratory tract infection, with HF as a secondary diagnosis were not included.

2.1.3 Chronic HF patients

Patients with a prior diagnosis of stable chronic HF were recruited from the outpatient clinics of the Glenfield Hospital and Leicester Royal Infirmary.

2.1.4 Exclusion criteria

Patients with a known life expectancy of less than 6 months or surgery in the previous month were excluded from the study.

2.2 Definition and determination of end points

The primary endpoints of the study were death from any cause or readmission to hospital with decompensated HF at 6 months (180 days). Readmission to hospital with decompensated HF was defined as a hospitalisation for which HF as defined as:

1. History of worsening shortness of breath
2. Compatible clinical signs of pulmonary rales or elevated JVP or peripheral oedema
3. HF as the primary diagnosis at admission

Death or readmission to hospital with decompensated HF was confirmed through the hospital electronic records and review of the office of national statistics registry. Secondary endpoints were death from any cause at 6 months and readmission to hospital with decompensated HF at 6 months individually.

2.3 Sample collection and storage

A blood and urine sample was taken from patients admitted to hospital with acute HF at admission and then again following treatment when the patient's condition was stable. Blood samples were taken after 15 minutes of bed rest to reduce potential variation in biomarkers due to posture and stress which for example is known to occur with cortisol and renin/aldosterone [172, 173]. 20 ml of blood was collected into tubes containing EDTA and aprotinin. Blood samples were immediately immersed in ice water and then centrifuged for 15 minutes at 3000 rpm at 10 °C. Plasma was then stored at −70 °C until assayed in a blinded fashion in a single batch. 50 ml urine was collected from each study subject into sterile tubes and stored at −70 °C. A single blood and urine sample was collected from the healthy controls and patients with chronic stable HF. 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 HF [174]. Units of eGFR are ml·min^{-1}1.73 m^{-2}.

2.4 Transthoracic ECHO

Transthoracic ECHO was performed on study subjects using an IE33 instrument (Philips Medical Systems, Reigate, UK). Left ventricular EF was calculated using the biplane method of discs formula. Impaired LV systolic function was defined as an EF<40%. Impairment of diastolic function was assessed using transmitral inflow velocity and mitral annular velocity. Pulse wave Doppler was used to measure the early diastolic inflow E wave and late diastolic inflow A wave. Tissue Doppler was used to measure the diastolic velocity é at the medial and lateral edge of the mitral valve annulus which were then used to calculate medial and lateral E/é ratios. The medial and lateral E/é ratios were averaged to give an overall measure of diastolic dysfunction.

2.5 Principles of immumoassay

Enzyme linked immunosorbent assay (ELISA) was the method used for the quantification of peptides. ELISA is a family of techniques developed simultaneously by Perlmann and Engvall in Sweden and Schuurs and van Weemen in the Netherlands in the early 1970's [175]. The double antibody sandwich variant of this method, in which two different antibodies specific for different epitopes on the peptide being measured, was used to measure ProUGN in the chronic HF cohort and NTproBNP in the acute HF cohort is briefly summarised as follows [176].

The bases of plate wells are coated with the first antibody to the peptide being measured, which acts as a capture antibody to immobilise the peptide (antigen) to the solid phase. The plate is then washed to remove excess unbound antibody, and then the test sample containing the peptide being measured is added. After further washing to remove excess unbound peptide, the second specific antibody is added, allowing the formation of 'antibody sandwiches'. This second antibody has previously been biotinylated, whereby the molecule biotin has been attached [177] and acts as the 'detection' antibody. Biotin has high affinity for the protein streptavidin but does not interfere with binding of the antibody to the peptide. The final step is the addition of streptavidin which has been conjugated with methyl acridinium ester (MAE). The streptavidin-MAE complex binds to the second antibody via the biotin molecule acting as a non-covalent bridge. MAE undergoes a chemiluminescent reaction under alkaline conditions following the addition of hydrogen peroxide resulting in the emission of light at a wavelength of 430 nm, detectable by a luminometer. The intensity of light detected is proportional to the concentration of the peptide in the test sample, and can be quantified by comparison to a standard curve produced from samples with known concentrations.

2.6 Materials

2.6.1 Antibodies

ProGN

The ProGN assay was performed using antibodies and standards from Biovendor, Brno, Czech Republic. Briefly, polyclonal antibodies to ProGN are coated onto ELISA plates. 20 µl of ProGN of plasma was pipetted into the ELISA plate wells together with standards up to 20 ng·ml^{-1}. After incubation for 1 h at room temperature, the plates were washed, and then the conjugate second polyclonal antibody pipetted into the wells for another 1 h of incubation. The ProGN antibody was conjugated with horseradish peroxidase, and subsequent detection used a colorimetric substrate (3, 3', 5, 5'-tetramethylbenzidine) measured on a MLX plate luminometer (Dynex Technologies Ltd, Worthing, UK) microplate reader using absorbance at 450 nm. The lower limit of detection for ProUGN was 0.45 ng·ml^{-1} with an interassay coefficient of variation 7.9% at 13 ng·ml^{-1} ProGN, n=8. The recovery of ProGN at 5 ng·ml^{-1} was 93.6%.

ProUGN

Two different ProUGN assays were used in this study. For the chronic HF cohort, the ProUGN was performed using antibodies and standards from Biovendor, Brno, Czech Republic. 50 µl of plasma was pipetted into the plate wells together with appropriate standards up to 20 ng·ml^{-1} respectively. After incubation for 1 h at room temperature, the plates were washed, and then the biotinylated second polyclonal antibody pipetted into the wells for another 1 h of incubation. The ProUGN antibody was biotinylated, and the subsequent detection step used the chemiluminescent MAE labelled streptavidin reaction under alkaline conditions detected using a MLX plate luminometer. The lower limit of detection for ProUGN was 0.65 ng·ml^{-1}. Recovery of ProUGN at 5 ng·ml^{-1} was 101.8% with an interassay coefficient of variation of 3.5% at 2 ng·ml^{-1}, n=8.

In between recruitment for the acute HF and the chronic HF studies, the ProUGN antibodies and standards manufactured by Biovendor, Brno, Czech

Republic, were withdrawn from sale, as demand for the reagents had been low. It was therefore necessary to develop a new ProUGN assay to measure the plasma levels in the chronic HF study.

The ProUGN assay employed in the acute HF study was a 1 site competitive assay, using plasma extracted on C18 solid phase extraction cartridges as an initial concentrating and interference removal step. Two monoclonal antibodies to the ProUGN sequence were a gift from Dr M Gani (Unipath, Bedford, UK). These were raised in rats against amino acids 44-58 of the preproUGN sequence (LSDLEAQWAPSPRLQ). The tracer was synthesised by biotinylation of the pure peptide (100 nmol) using maleimide-PEG2-biotin (200 nmol, from Pierce, Thermo Scientific, Northumberland, UK) and purifying the tracer on High performance liquid chromatography using a reversed phase C18 column and a linear acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Plasma samples (0.3 ml) were acidified by mixing with 2 ml of 0.1% TFA. Samples were loaded onto C18 solid phase extraction columns (Waters, Manchester). Unbound material and interferences were washed off using 3 x 2 ml 0.1% TFA washes, followed by another wash with 10% acetonitrile in 0.1% TFA. The ProUGN was eluted using 60% acetonitrile in 0.1% TFA and the volatile solvents removed in a vacuum centrifuge. The remaining solvent (mainly water) was removed by freeze drying the samples.

Preliminary studies were performed using some remaining ProUGN full-length standard to ensure the extraction procedure recovered this peptide. Plasma was extracted as above, and elutions at 25% and 60% acetonitrile were assayed for ProUGN. In addition, the zero and 5 $ng{\cdot}ml^{-1}$ ProUGN standards (approximately 125 fmol) were also spiked into plasma and the 60% acetonitrile fractions assayed for ProUGN recovery. There was good recovery of the ProUGN standard in the 60% acetonitrile fractions, with little in the 25% acetonitrile fraction. The zero standard showed a background signal only. The small signal from the unspiked plasma 60% acetonitrile fractions may represent endogenous ProUGN present in the plasma samples.

The non-competitive assay was performed by coating microlite-2 ELISA plates with 100 ng of the ProUGN monoclonal antibody, dissolved in PBS (phosphate buffered saline). After an overnight coating, plates were washed, and

then blocked using 0.1% bovine serum albumin in PBS for 2 h. Samples were reconstituted in ILMA buffer. 100 μl of sample (equivalent to 100 μl plasma) and standards (ranging from 1.95 to 2000 fmol per well) were pipetted into the antibody coated wells, and incubated overnight at 4 °C. The following day the tracer was added to the wells (250 fmol in 50 μl of ILMA) and incubated for another 24 h at 4 °C. The plates were then washed, and bound biotinylated tracer measured using methyl-acridinium labelled streptavidin, with chemiluminescence determined as described above. The lower limit of detection for this assay was 8.9 $pmol \cdot l^{-1}$.

cGMP

cGMP was measured using a 1 site competitive enzyme immunoassay from R&D systems, Minneapolis, USA. 100 μl of diluent for plasma was added to the zero standard wells and 150 μl to the non-specific binding wells. 5 μl of plasma was pipetted into the remaining wells with 95 μl of diluent together with appropriate standards. 50 μl of cGMP conjugate was added to each well followed by 50 μl of primary antibody solution, excluding the non-specific binding wells. After incubation at room temperature for 4 h, the plates were aspirated and washed 4 times. 200 μl of substrate solution was added to each well and incubated for 30 mins in the dark. Finally 50 μl of stop solution was added to each well and the plates read using a Multiskan Ascent luminometer (Thermo Fisher Scientific Corps, Massachusetts, USA) at 430 nm.

NTproBNP

Plasma NTproBNP was measured using our in-house non-competitive assay described in detail in [178] and [179]. Sheep antibodies were raised to the N-terminal of human NT-proBNP and monoclonal mouse antibodies were raised to the C- terminal. The N-terminal IgG was affinity-purified and biotinylated. Samples or NTproBNP standards were incubated in C-terminal IgG coated wells with the biotinylated antibody for 24 h at 4 °C. Detection was with MAE labelled streptavidin. The lower limit of detection was 0.3 $pmol \cdot l^{-1}$. There was no cross-reactivity with atrial natriuretic peptide, BNP or C-type natriuretic peptide.

Inter- and intra- coefficients of variation were 2.3% and 4.8% respectively. The results from this in-house assay are highly correlated (r=.90, p<.0001, n=86) to those obtained in the NT-proBNP Elecys assay (Roche Diagnostics).

2.6.2 Stock solutions and buffers

Constituents of ILMA buffer:

- NaH_2PO_4 1.5 mmol·l^{-1}
- Na_2HPO_4 8 mmol·l^{-1}
- NaCl 140 mmol·l^{-1}
- EDTA 1.0 mmol·l^{-1}
- BSA 1.0 g·l^{-1}
- Azide 0.1 g·l^{-1}
- Triton X100 0.1%
- pH 7.4

Constituents of wash buffer B:

- Tween 0.05 %
- NaCl 118.5 g
- Na_2HPO_4 6.4 g
- NaH_2PO_4 1.4 g
- Na Azide 6.1 g·l^{-1}
- pH 7.4

Constituents of wash buffer C: Phosphate buffered solution (PBS)

- NaCl 137 mmol·l^{-1}
- KCl 2.7 mmol·l^{-1}
- Na_2HPO_4 8 mmol·l^{-1}
- KH_2PO_4 1.5 mmol·l^{-1}

2.7 Sample size calculation

Assuming that we wish to detect differences for ProUGN and the ProUGN/cGMP ratio at a p value corrected for multiple testing of .05, the sample size of 312 would have a power of 80% or more to detect a standardized hazard ratio of 4 using Cox survival analysis, assuming that all other covariates may account for not more than 30% of the variance of ProUGN and the ProUGN/cGMP ratio, the covariate SD was 0.25 and that the prevalence of the combined endpoint death and heart failure was 30%. This combined endpoint estimate reflects the high mortality and readmission rate of an acute HF population. Sample size calculation was based on the method of Hsieh and Lavori [180], as implemented in the software package Stata 10.0, Texas. This sample size will also enable a standardised difference of ProUGN and the ProUGN/cGMP ratio between admission and discharge, of 0.2 to be detected at p=.01 with a power of 82%. For comparisons with normal controls and chronic HF patients, the samples sizes of 24 and 78 will enable a standardised difference of 1 to be detected with a power of 95% at p=.01.

2.8 Statistical analysis

R 2.12 [181] was used to conduct statistical analyses. Continuous variables were expressed as median [interquartile range (IQR)] as this describes the distribution as well as reducing the influence of outliers. Parametric methods were used in preference to non-parametric methods as the dataset was large and thus the central limit theorem applies to the distribution of the means of continuous data. Continuous variables with skewed distributions were $\log_{10}$ transformed prior to analysis. The Bonferroni correction was used to correct for multiple comparisons. Binary classification was assessed by receiver operating characteristic (ROC) curves with calculation of area under curve (AUC) as derived by the method of Hanley and McNeil [182]. The difference between stratified Kaplan-Meier plots was assessed using the Log rank test. A 2-tailed p value of $<.05$ was deemed to be statistically significant, with p values given to 3 decimal places.

Chapter 3

Chronic heart failure

3.1 Baseline characteristics

243 consecutive patients with chronic HF referred to a tertiary referral cardiology centre were recruited, along with 72 healthy controls. Demographic features of the HF and control groups are shown in Table 3.1.

HF patients were significantly older than the healthy controls (70 [58 to 79] vs 65 [60 to 70] years, p=.018), while their eGFR was significantly worse (51 [42 to 69] vs 72 [62 to 79], p<.001). The EF of the HF patients (35 [26 to 42]%) was significantly worse than in the healthy controls (62 [57 to 65]%), p<.001 (Figure 3.1).

ProUGN levels were significantly greater in HF patients (872 [600 to 1428] pmol·l^{-1}) compared to healthy controls (665 [518 to 855] pmol·l^{-1}) p<.001, and similarly ProGN levels were significantly greater in HF patients (763 [525 to 1232] pmol·l^{-1}) compared to healthy controls (600 [452 to 698] pmol·l^{-1}), p<.001 (Figure 3.1). There was still a significant difference in ProGN and ProUGN levels between the healthy controls and chronic HF patients when age and eGFR were included as covariates using ANCOVA, p<.001 for both. ProGN and ProUGN both showed strong positive correlations with each other in healthy controls (r=.59 [.41 to .72], p<.001) as well as HF patients (r=.78 [.73 to .83], p<.001).

Table 3.1 Comparison of baseline characteristics of healthy controls and chronic HF patients

	Control[a] (n = 72)	**HF patients**[a] (n = 243)	**p**[b]
Age (years)	65 (60 to 70)	70 (58 to 79)	.018
Male sex	43 (59.7%)	151 (62.1%)	.897
eGFR[c]	72 (62 to 79)	51 (42 to 69)	<.001
Peptide biomarkers			
ProUGN (pmol·l^{-1})	665 (518 to 855)	872 (600 to 1428)	<.001
ProGN (pmol·l^{-1})	600 (452 to 698)	763 (525 to 1232)	<.001
NTproBNP (pmol·l^{-1})	42.4 (5.7 to 99.0)	1268 (327 to 5249)	<.001
Echo parameters			
EF (%)	62 (57 to 65)	35 (26 to 42)	<.001
LVIDD (cm)	4.5 (4.2 to 4.8)	6.0 (5.3 to 6.7)	<.001
Past medical history			
Hypertension	None	109 (44.9%)	–
Myocardial infarction	None	92 (37.9%)	–
Diabetes	None	43 (17.7%)	–
AF	None	49 (20.2%)	–
Medications			
Diuretic	None	192 (79.0%)	–
Betablocker	None	97 (39.9%)	–
ACEi	None	163 (67.1%)	–
CCB	None	28 (11.5%)	–

[a] Values are median (IQR) or n (% of n)
[b] t test or chi squared test
[c] Units (ml·min^{-1}1.73 m^{-2})

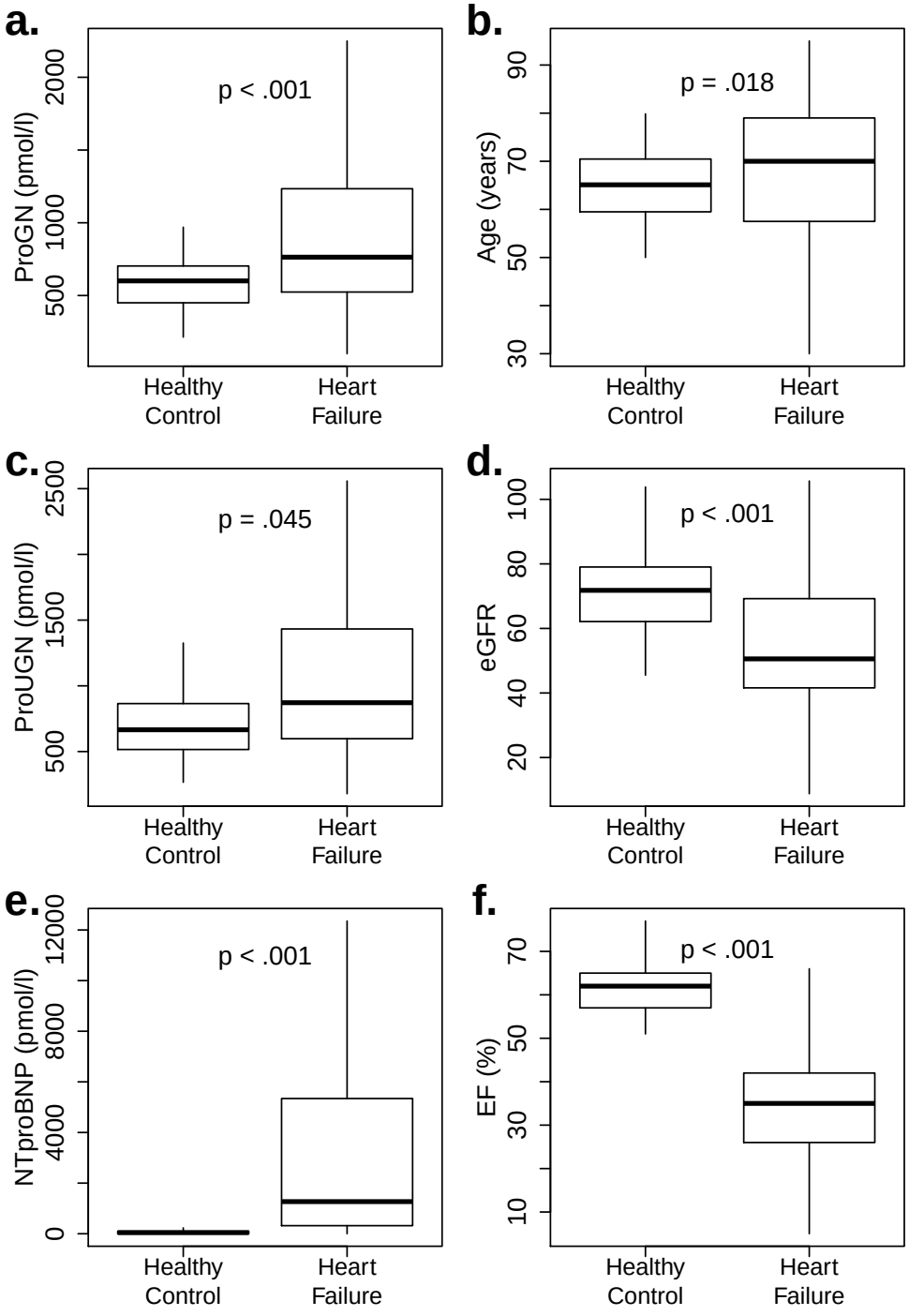

Figure 3.1 Comparison of baseline characteristics of healthy controls and HF patients. Plasma ProGN, ProUGN and NTproBNP levels were significantly greater in HF patients compared to controls, while HF patients were significantly older and had a worse eGFR and EF. p values are shown for the t test.

3.2 Peptide levels in healthy controls

In the healthy controls there was no significant difference between ProUGN or ProGN levels in males and females (642 [514 to 805] vs 759 [542 to 899] pmol·l^{-1}, p=.164) and (600 [456 to 694] vs 601 [445 to 712] pmol·l^{-1}, p=.309) respectively.

Correlations between ProGN and ProUGN levels and continuous variables in healthy controls are shown in Figure 3.2. ProGN was not significantly correlated with eGFR (r=−.23 [−.48 to .05], p=.105) or EF (r=−.10 [−.43 to .25], p=.589) but was significantly correlated with age (r=.32 [.09 to .51], p<.001). ProUGN showed a similar pattern of correlations to ProGN, being positively correlated with age (r=.20 [−.04 to .41], p=.100) and inversely correlated with EF (r=−.12 [−.45 to .23], p=.494) and significantly inversely correlated with eGFR (r=−.37 [−.59 to −.10], p=.010).

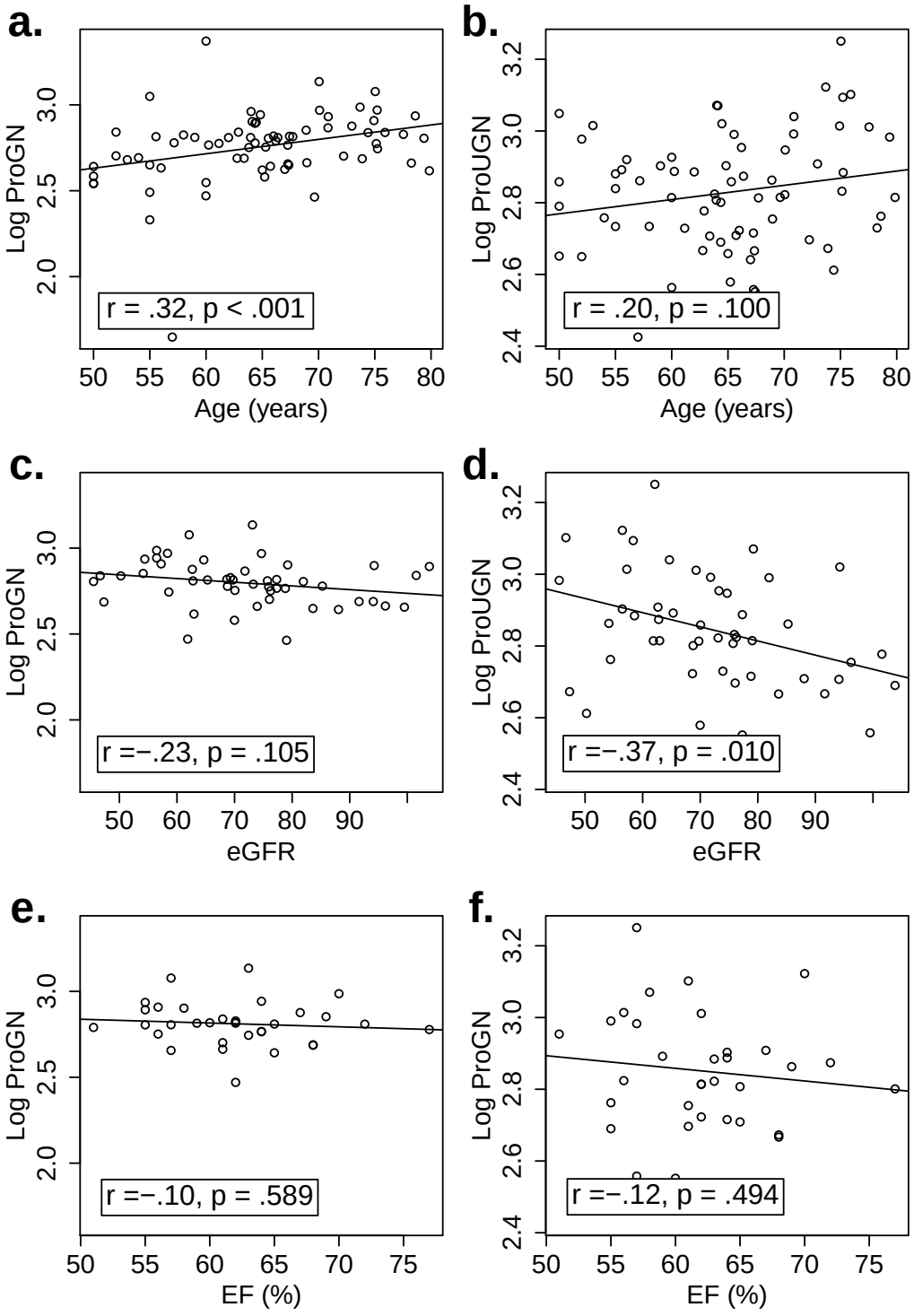

Figure 3.2 Scatter plots of ProGN and ProUGN in healthy controls. ProGN was significantly positively correlated with age while ProUGN was significantly inversely correlated with eGFR. Pearson's r coefficient and p values are shown in the text boxes.

3.3 Peptide levels in HF patients

3.3.1 Univariate analysis of peptide levels

The relationships between ProUGN and ProGN levels and dichotomous variables in HF patients are shown in Tables 3.2 and 3.3.

Plasma ProUGN was significantly greater in patients with a history of hypertension (1151 [684 to 1754] vs 728 [561 to 1188] pmol·l^{-1}, p<.001), diabetes (1181 [737 to 1923] vs 852 [589 to 1329] pmol·l^{-1}, p=.021), AF (1242 [718 to 2075] vs 855 [592 to 1328] pmol·l^{-1}), p=.002) as well as in those prescribed diuretics (1007 [640 to 1525] vs 689 [579 to 922] pmol·l^{-1}), p=.005) compared to those without these characteristics (Figures 3.3 3.4 and 3.5).

As with ProUGN, plasma ProGN was significantly greater in patients with a history of hypertension, (908 [621 to 1428] vs 665 [490 to 1096] pmol·l^{-1}, p=.001), diabetes (1048 [667 to 1455] vs 737 [514 to 1136] pmol·l^{-1}, p=.010), AF (1089 [720 to 1441] vs 712 [505 to 1136] pmol·l^{-1}, p=.003), as well as in those prescribed diuretics (859 [578 to 1362] vs 545 [429 to 763] pmol·l^{-1}, p<.001) compared to those without these characteristics (Figures 3.3 3.4 and 3.5).

Correlations between ProGN and ProUGN levels and continuous variables in HF patients are shown in Figures 3.6. ProGN was significantly correlated with eGFR (r=−.44 [−.56 to −.29], p<.001), EF (r=.26 [.05 to .45], p=.015), age (r=.37 [.25 to .47], p<.001) but not LVIDD (r=−.05 [−.25 to .16], p=.659). ProUGN showed a similar pattern of correlations to ProGN, being positively correlated with age (r=.31 [.19 to .43], p<.001), EF (r=−.09 [−.13 to −.29], p=.428) and significantly inversely correlated with eGFR (r=−.45 [−.57 to −.31], p<.001) but not LVIDD (r=−.10 [−.30 to −.10], p=.333).

Age was inversely correlated with eGFR, (r=−0.48 [−0.60 to −0.35], p<.001).

Comparison of ProGN and ProUGN correlations with age,eGFR and EF between healthy controls and HF patients are shown in Figures 3.7.

Table 3.2 Univariate analysis of plasma ProUGN in HF patients

	ProUGN[a] **(pmol·l^{-1})**	**p**[b]
Males vs females	824 (577 to 1329) vs 1046 (670 to 1444)	.142
Past medical history vs none		
MI	870 (607 to 1362) vs 967 (593 to 1492)	.854
Hypertension	1151 (684 to 1754) vs 728 (561 to 1188)	<.001
Diabetes	1181 (737 to 1923) vs 852 (589 to 1329)	.021
AF	1242 (718 to 2075) vs 855 (592 to 1328)	.002
Drug history vs none		
Diuretic	1007 (640 to 1525) vs 689 (579 to 922)	.005
Betablocker	745 (575 to 1433) vs 976 (635 to 1386)	.282
ACEi	903 (595 to 1542) vs 727 (629 to 1249)	.156
CCB	693 (545 to 1330) vs 797 (579 to 1161)	.916

[a] Median (IQR)
[b] t test of log values

Table 3.3 Univariate analysis of plasma ProGN in HF patients

	ProGN[a] **(pmol·l^{-1})**	**p**[b]
Males vs females	749 (511 to 1227) vs 773 (584 to 1235)	.751
Past medical history vs none		
MI	754 (559 to 1283) vs 830 (540 to 1235)	.930
Hypertension	908 (621 to 1428) vs 665 (490 to 1096)	.001
Diabetes	1048 (667 to 1455) vs 737 (514 to 1136)	.010
AF	1089 (720 to 1441) vs 712 (505 to 1136)	.003
Drug history vs none		
Diuretic	859 (578 to 1362) vs 545 (429 to 763)	<.001
Betablocker	745 (510 to 1135) vs 804 (547 to 1328)	.131
ACEi	826 (547 to 1346) vs 712 (522 to 1023)	.078
CCB	571 (503 to 1023) vs 661 (493 to 1103)	.737

[a] Median (IQR)
[b] t test of log values

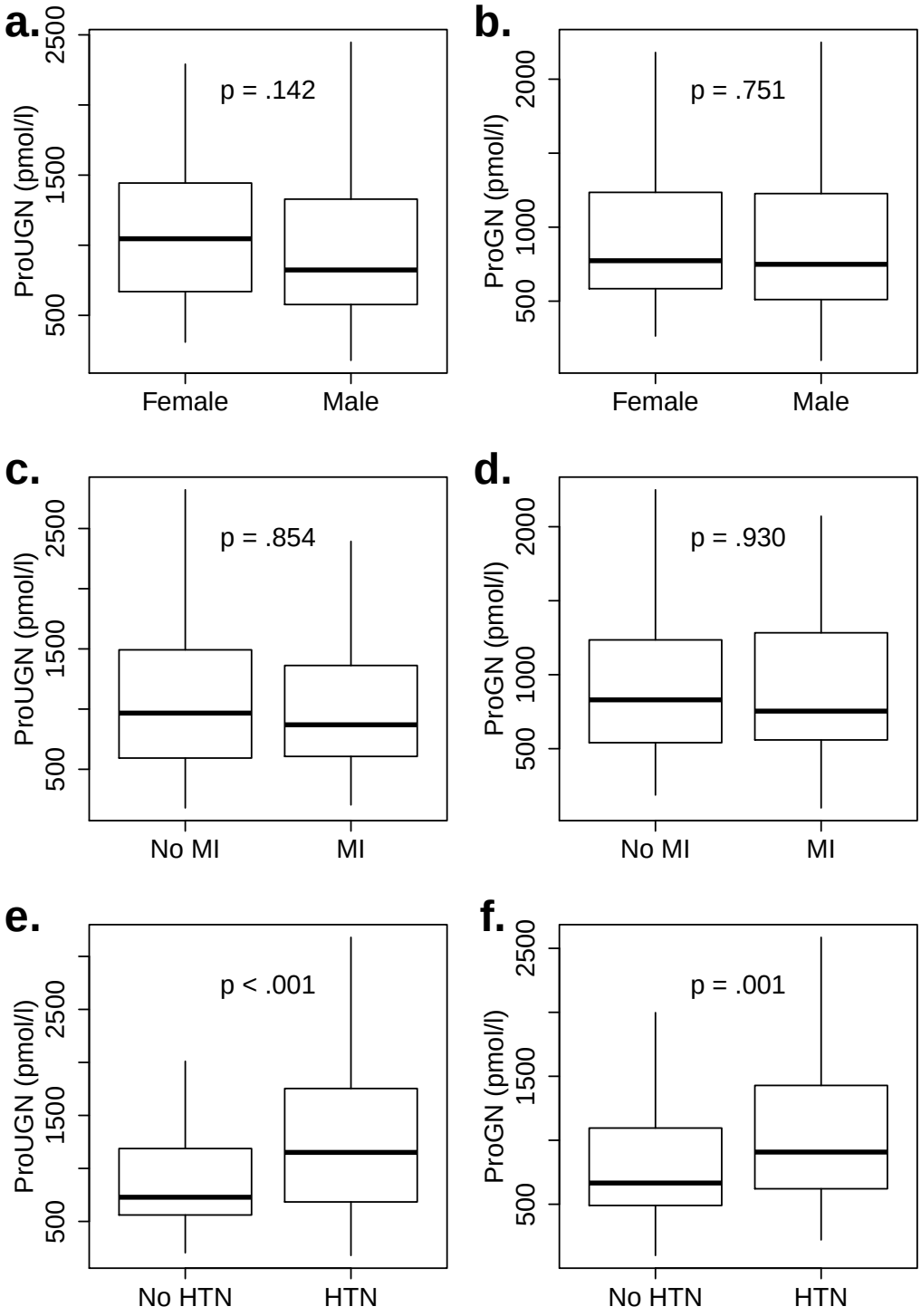

Figure 3.3 Box plots of ProUGN and ProGN in HF patients. ProUGN and ProGN were both significantly greater in patients with hypertension compared to without. p values are shown for the t test.

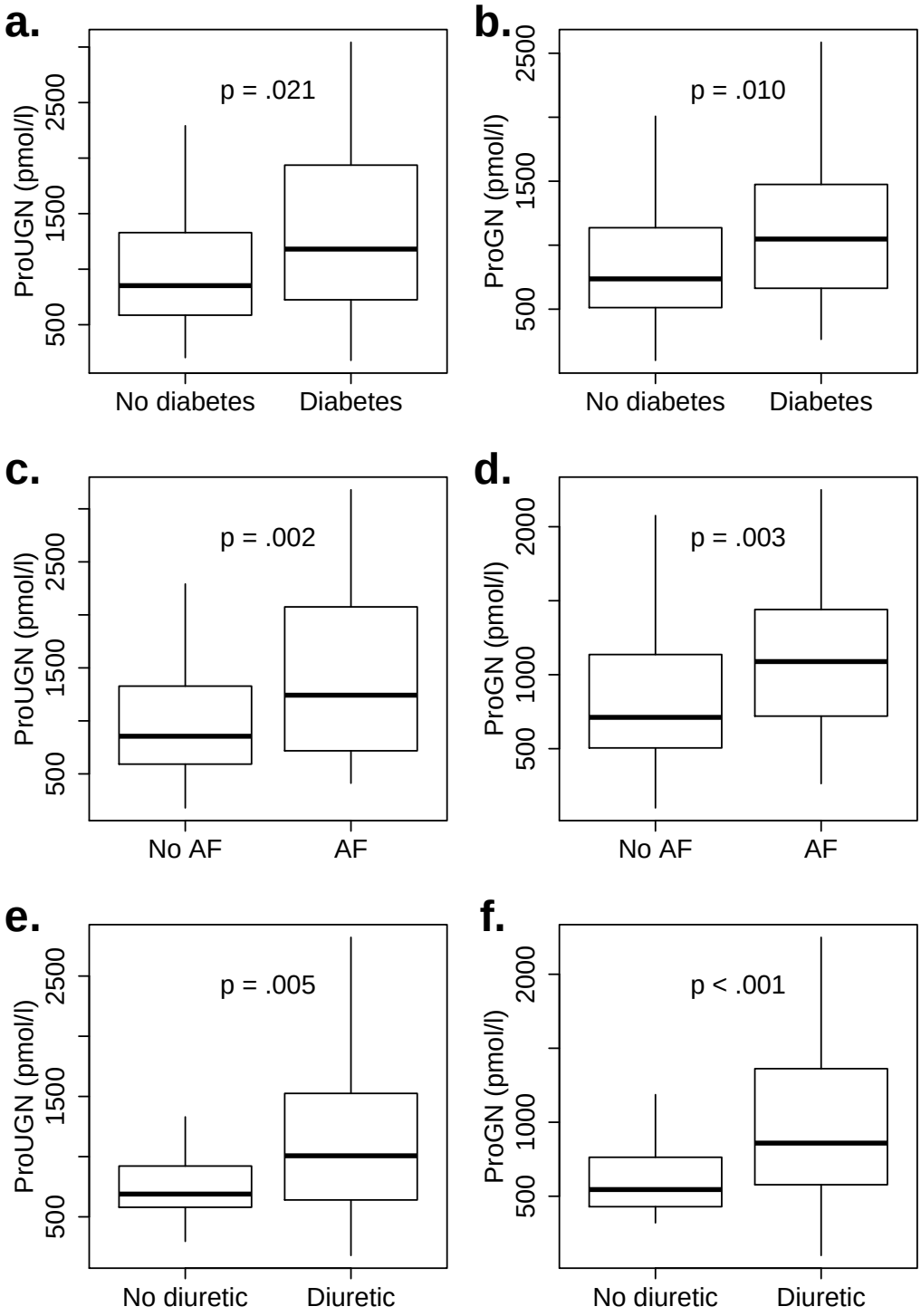

Figure 3.4 Box plots of ProUGN and ProGN in HF patients. ProUGN and ProGN were both significantly greater in patients with diabetes and AF as well as those taking diuretics compared to those not. p values are shown for the t test.

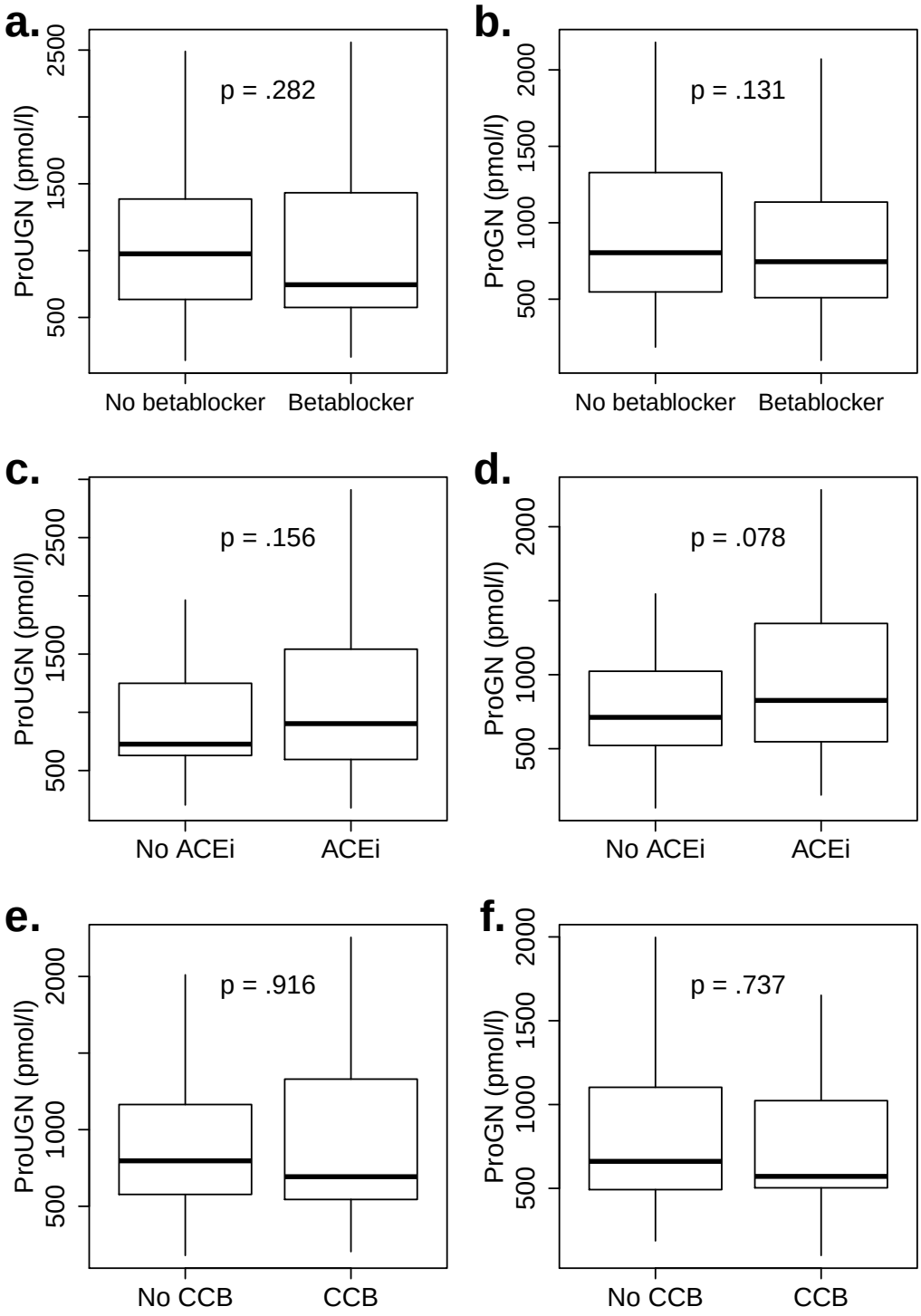

Figure 3.5 Box plots of ProUGN and ProGN in HF patients. There were no significant differences in ProUGN and ProGN levels in those taking betablockers, ACEi or CCBs. p values are shown for the t test.

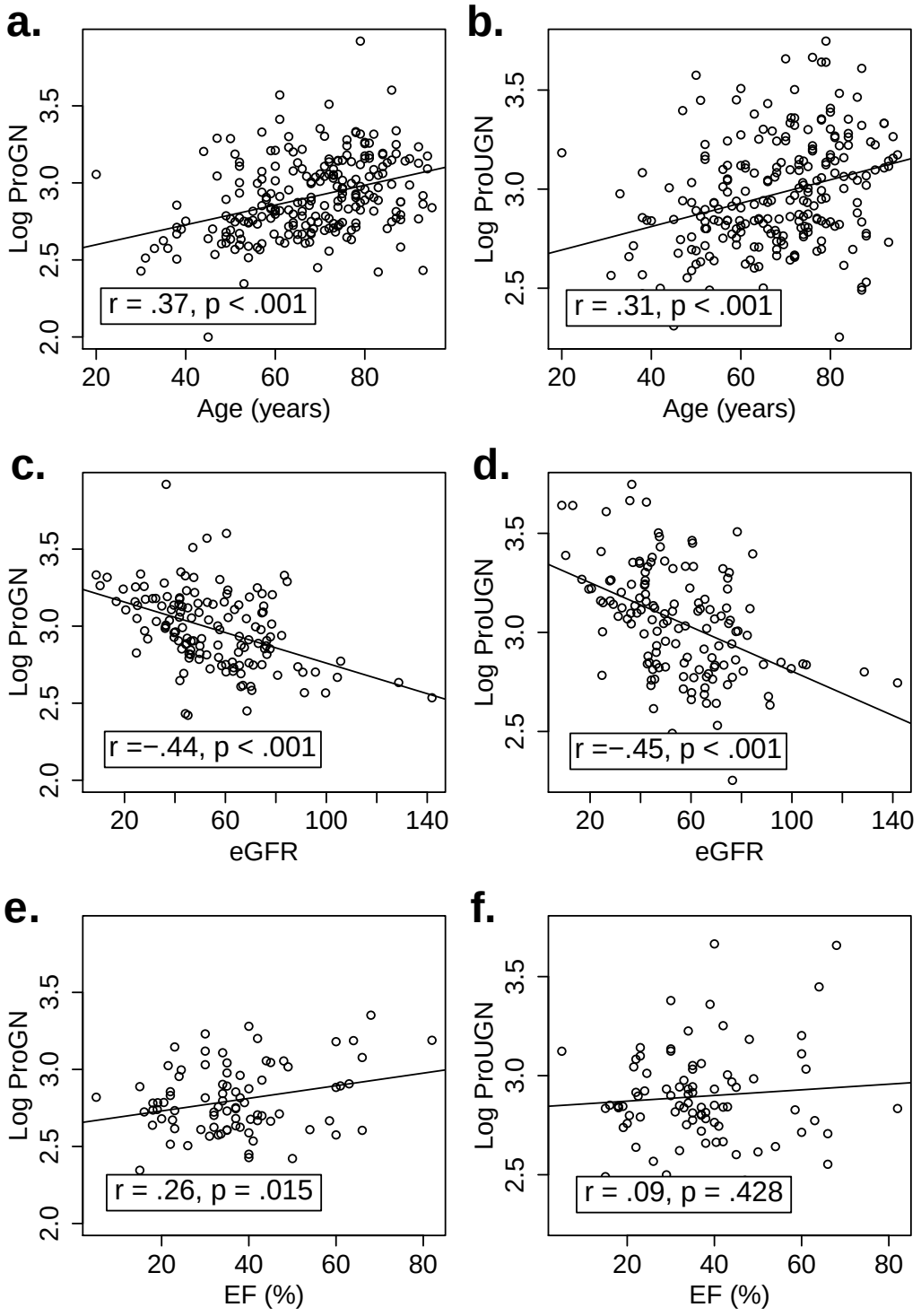

Figure 3.6 Scatter plots ProGN and ProUGN in HF patients. ProGN and ProUGN were significantly positively correlated with age and inversely correlated to eGFR. ProGN was significantly positively correlated with EF. Pearson's r coefficient and p values are shown in the text boxes.

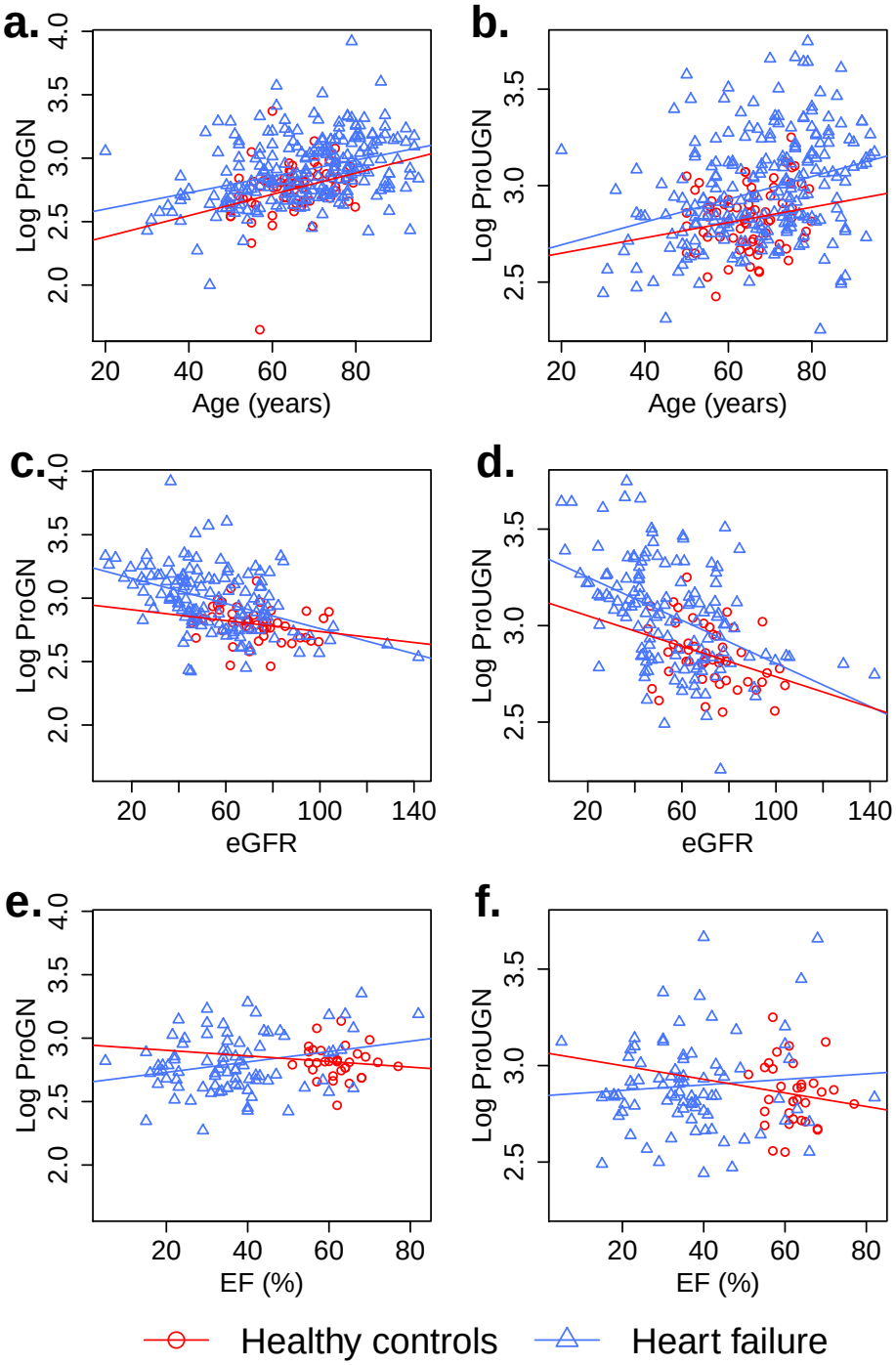

Figure 3.7 Scatter plots comparing ProGN and ProUGN correlations with age,eGFR and EF in healthy controls and HF patients.

3.3.2 Peptide levels by severity of HF

Figures 3.8 show the the variation of ProUGN and ProGN by NYHA class and LV impairment in HF patients compared to healthy controls.

One way ANOVA showed an overall significant difference between NYHA class for both ProUGN and ProGN ($p<.001$). Pairwise comparisons performed by Student t tests using Bonferroni's correction showed a significant difference between ProUGN levels in those in NYHA class IV compared to controls ($p<.001$) and NYHA class I ($p=.014$) as well as between those in NHYA class III and controls ($p<.001$), NYHA class II ($p=.011$) and NHYA class I ($p=.002$). Pairwise comparisons showed a significant difference in ProGN level in NYHA classes III and IV versus controls ($p<.001$ for both), NYHA classes III and VI versus class I ($p<.001$ for both) and NYHA class III and IV versus class II ($p=.016$ and $p=.010$ respectively).

There was an overall significant difference between ProGN levels by LV functional status ($p<.001$) but not ProUGN ($p=.097$). Pairwise comparisons showed a significant difference in ProGN levels between those with severe impairment versus normal function ($p<.001$) and versus mild impairment ($p=.001$). There was no significant differences in ProUGN levels between LV functional status groups.

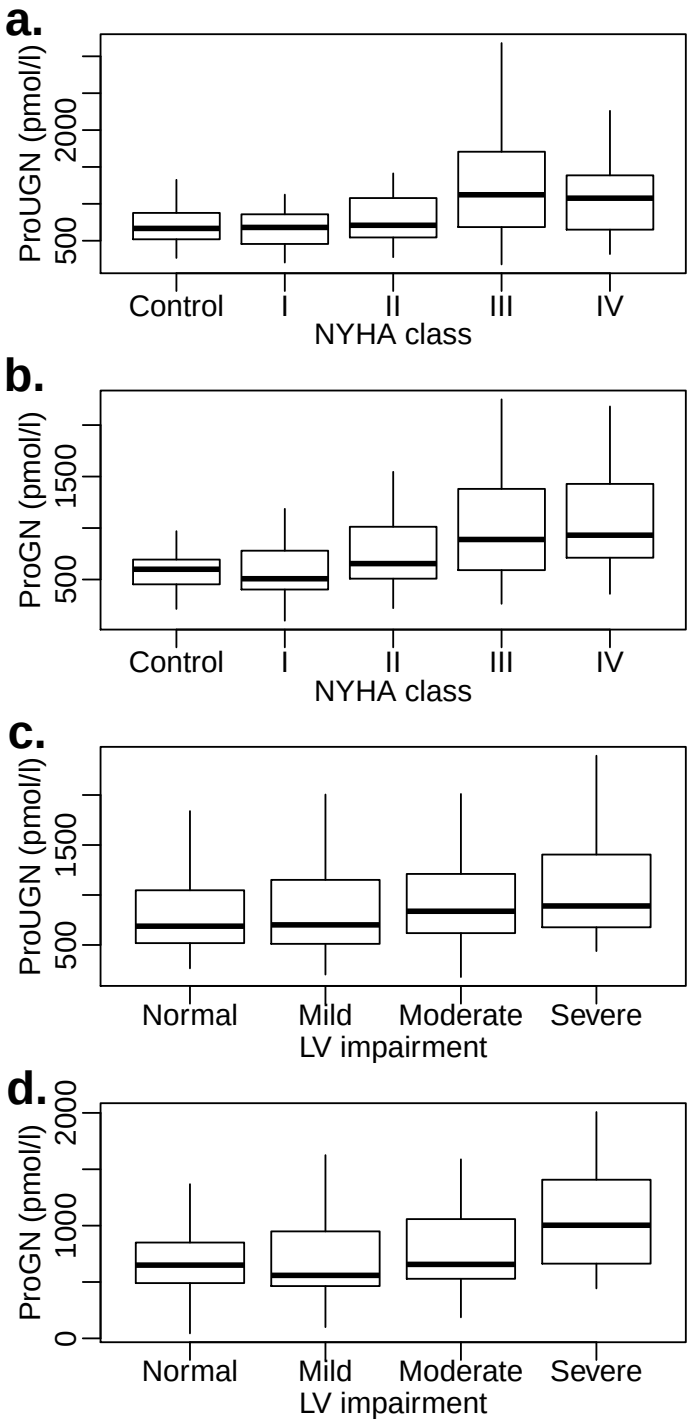

Figure 3.8 ProUGN and ProGN levels by NYHA class and LV functional status. p values for one way ANOVA for between group differences are **a.** p<.001 **b.** p<.001 **c.** p=.097 **d.** p<.001.

3.3.3 Linear analysis

Variables accounting for the variation in ProUGN and ProGN levels were identified using linear regression. Variables significant in univariate analysis were entered into a multivariate model to identify independent associations, shown in Tables 3.4 and 3.5. Increasing age, a history of hypertension, diabetes, AF and diuretic use were all significantly associated with increased ProUGN and ProGN levels, while decreased eGFR was associated with increased ProUGN and ProGN in univariate analysis. When entered into a multivariate model, only a decreased eGFR remained independently associated with increased plasma ProUGN and ProGN.

Table 3.4 Linear regression of plasma ProUGN[a] in HF

	Univariate			Multivariate		
	B[b]	**95% C.I.**	**p**	**B**[b]	**95% C.I.**	**p**
Age	5.90	3.58 *to* 8.26	<.001	−2.05	−6.15 *to* 2.05	.324
Sex	−55.1	−129 *to* 18.6	.142			
LVIDD	−1.98	−6.04 *to* 2.06	.333			
eGFR	−5.59	−7.47 *to* −3.70	<.001	−5.95	−8.27 *to* −3.63	<.001
Past medical history						
MI	−7.30	−85.6 *to* 71.0	.854			
Hypertension	135	65.7 *to* 205	<.001	25.5	−64.2 *to* 115	.574
Diabetes	110	17.1 *to* 203	.021	−0.74	−107 *to* 106	.989
AF	141	53.6 *to* 228	.002	69.6	−24.9 *to* 164	.148
Medication history						
Diuretic	126	39.1 *to* 213	.005	−11.3	−143 *to* 121	.866
Betablocker	−39.8	−112 *to* 32.8	.282			
ACEi	55.2	−21.2 *to* 132	.156			
CCB	−5.91	−116 *to* 104	.916			

[a] Log value
[b] Values are $\times 10^{-3}$

Table 3.5 Linear regression of plasma ProGN[a] in HF

	Univariate			Multivariate		
	B[b]	**95% C.I.**	**p**	**B**[b]	**95% C.I.**	**p**
Age	6.44	4.31 *to* 8.56	<.001	−2.14	−5.89 *to* 1.60	.258
Sex	−11.2	−80.3 *to* 58.0	.750			
LVIDD	−0.86	−4.70 *to* 2.98	.659			
eGFR	−5.00	−6.68 *to* −3.23	<.001	−4.56	−6.68 *to* −2.44	<.001
Past medical history						
MI	3.19	−68.8 *to* 75.1	.930			
Hypertension	110	44.9 *to* 176	.001	1.72	−80.2 *to* 83.6	.967
Diabetes	115	28.3 *to* 202	.001	19.7	−77.3 *to* 117	.689
AF	123	41.5 *to* 205	.003	31.5	−54.7 *to* 118	.472
Medication history						
Diuretic	172	91.6 *to* 251	<.001	104	−16.0 *to* 225	.089
Betablocker	−52.1	−120 *to* 15.7	.131			
ACEi	64.1	−7.17 *to* 135	.078			
CCB	−18.4	−126 *to* 89.6	.737			

[a] Log value
[b] Values are $\times 10^{-3}$

Chapter 4

Acute heart failure

4.1 Recruitment

336 patients admitted to hospital with acute HF were recruited in addition to 26 healthy controls. 336 blood samples were drawn from the HF patients recruited with the median time between hospital admission and the first blood sample ("Admission") being 1 day [range 0 to 3]. A second blood sample was taken in 214 of these patients when they had recovered pre-discharge. The median time between the first and second blood sample was 7 days [range 1 to 36]. Demographic data was collected for all HF patients and echocardiography was performed in 242.

4.2 Baseline characteristics

Baseline characteristics of HF patients and healthy controls are shown in Table 4.1. There was no significant difference in age, proportion of males or renal function between healthy controls and HF patients. 139 (41%) patients admitted with acute HF had a recorded previous history of HF. The primary aetiology of HF was recorded as IHD in 143 (42.6%), valvular heart disease in 62 (17.9%), hypertension in 60 (17.9%), idiopathic dilated cardiomyopathy in 12 (3.6%), alcoholic cardiomyopathy in 4 (1.2%), amyloid in 2, hypertrophic cardiomyopathy in 1, restrictive in 1, unknown in 38 (11.3%) and not recorded

in 13. 91 (27.1%) had a recorded previous history of a MI, 50 (14.9%) had underwent a CABG, 25 (7.4%) had undergone a PCI, 34 (10.1%) had a permanent pacemaker, 5 had a cardiac resynchronisation device and 4 had an implantable cardioverter-defibrillator.

The distribution of selected characteristics of the HF patients are shown in Figure 4.1. All patients reported breathlessness at admission concomitant with NYHA class III or IV. 232 (69%) reported a history of orthopnea, 148 (44%) of PND, while on examination 267 (79%) had pulmonary rales, 309 (92%) had peripheral oedema and 210 (63%) had an elevated JVP. Of 313 reported chest x-rays, 163 (52%) showed pulmonary oedema or upper lobe diversion and 137 (44%) had a pleural effusion. Of 231 patients who had a posterior-anterior chest x-ray, 209 (90%) had a cardiothoracic ratio > 0.5. Out of 218 patients with a measurement, 146 (67%) had an EF $< 40\%$ and of 225 patients with a measurement 111 (49%) had a LVIDD > 5.2 cm. Of 51 females with a measurement, 20 (39%) had an EDV > 105 ml, while of 161 males with a measurement, 46 (29%) had an EDV > 155 ml. In 330 admission ECGs the QRS duration was greater than 120 ms in 138 (42%) of HF patients. All patients had an NTproBNP greater than 115 pmol$\cdot$l^{-1}, a previously established diagnostic threshold for ventricular dysfunction in patients admitted with acute dyspnoea [183]. Out of 109 patients in whom tissue Doppler was performed, only 7 had an EF $> 50\%$ and E/é > 15 indicating predominantly diastolic HF.

Table 4.1 Baseline characteristics of healthy controls and HF patients

	Control[a] (n = 26)	**HF patients**[a] (n = 336)	**p**[b]
Age (years)	73 (71 to 76)	77 (69 to 83)	.656
Male sex	18 (69)	262 (78)	.309
BMI	25.4 (23.1 to 27.0)	31.5 (27.3 to 37.4)	<.001
eGFR[c]	57 (51 to 69)	54 (40 to 69)	.332
Admission biomarkers			
ProUGN (pmol·l^{-1})	3623 (2071 to 4553)	1297 (904 to 1763)	<.001
NTproBNP (pmol·l^{-1})	5.7 (0.3 to 53.9)	2942 (1439 to 5709)	<.001
cGMP (nmol·l^{-1})	129 (94 to 185)	109 (66 to 148)	.006
ECHO parameters			
EF (%)	–	35 (27 to 42)	–
LVIDD (cm)	–	5.2 (4.7 to 5.9)	–
LVIDS (cm)	–	4.4 (3.9 to 5.2)	–
Past medical history			
HF	None	139 (41%)	–
Hypertension	None	195 (58%)	–
IHD	None	143 (43%)	–
Diabetes	None	117 (35%)	–
Valve disease	None	62 (18%)	–
Hyperlipidaemia	None	81 (24%)	–
Smoker	None	162 (48%)	–
AF	None	127 (38%)	–
Asthma or COPD	None	69 (21%)	–
Medications			
Aspirin	None	147 (44%)	–
Diuretic	None	202 (60%)	–
Betablocker	None	184 (55%)	–
ACEi or ARB	None	201 (59%)	–
CCB	None	71 (21%)	–
ARA	None	35 (10%)	–
Statin	None	179 (53%)	–

[a] Values are median (IQR) or n (%n)
[b] t test or chi squared test
[c] Units (ml·min^{-1}1.73 m^{-2})

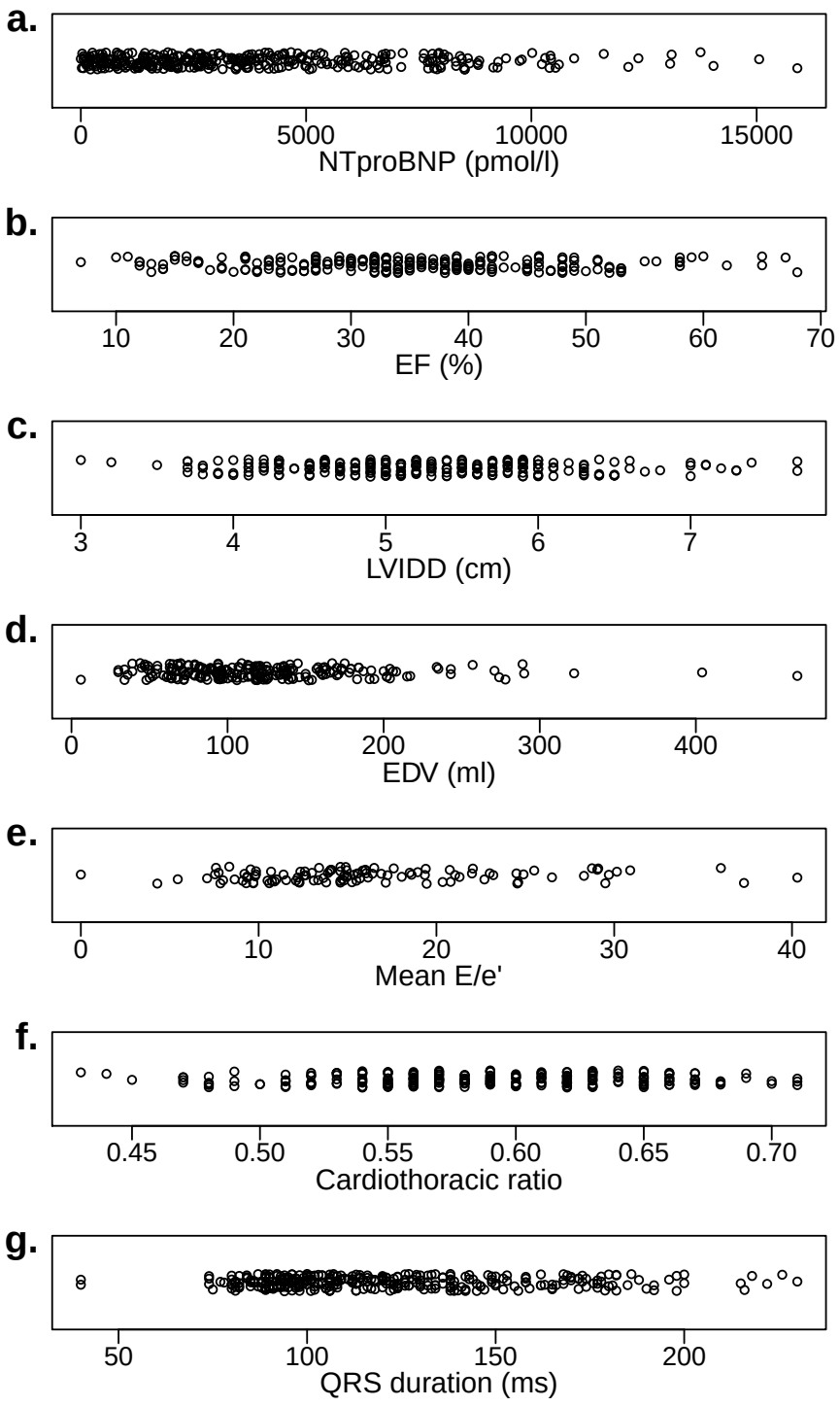

Figure 4.1 Strip charts for selected continuous variables in HF patients.

4.3 Admission and pre-discharge levels

Plasma levels of ProUGN, cGMP and NTproBNP at admission and pre-discharge compared to healthy controls are shown in Figure 4.2 and compared in Tables 4.1 and 4.2. Admission ProUGN and cGMP levels were significantly lower in HF patients compared to healthy controls, while admission NTproBNP was significantly greater. Pre-discharge NTproBNP levels were significantly lowered compared to admission but still elevated when compared to controls while pre-discharge cGMP levels were significantly greater than at admission while being lower but not significantly different than in healthy controls (p=.231). Pre-discharge ProUGN levels were significantly greater than at admission and also significantly greater than in the healthy controls (p<.001).

Table 4.2 Control, admission and pre-discharge biomarker levels

	Control[a]	**Admission**[a]	**Pre-discharge**[a]	**p**[b]
ProUGN[c]	3623 (2071 to 4553)	1297 (904 to 1763)	4740 (2983 to 7390)	<.001
NTproBNP[c]	5.7 (0.3 to 53.9)	2942 (1439 to 5709)	1848 (736 to 3869)	<.001
cGMP[d]	129 (94 to 185)	109 (66 to 148)	122 (85 to 181)	.045

[a] Values are median (IQR)
[b] Paired t test of log values Admission *vs* pre-discharge levels
[c] Units pmol·l^{-1}
[d] Units nmol·l^{-1}

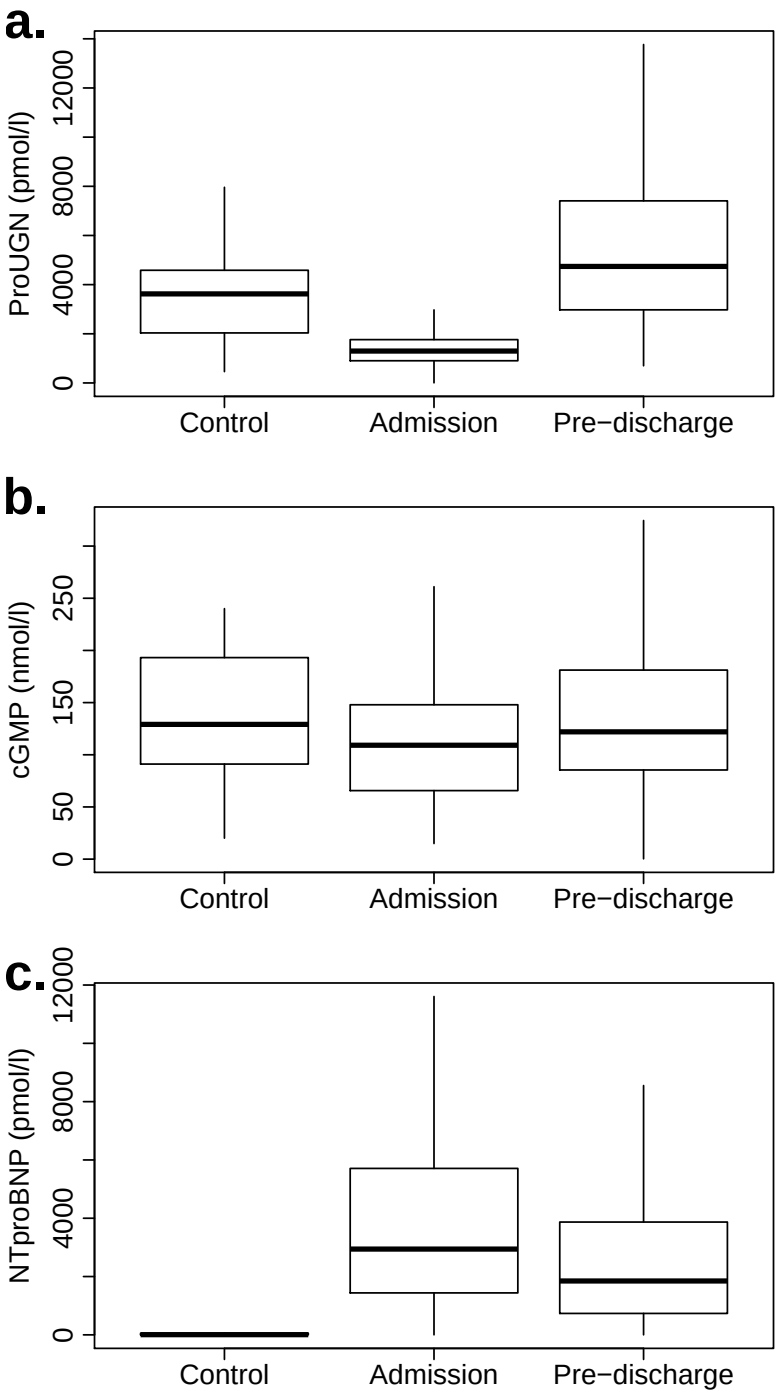

Figure 4.2 Box plots of ProUGN, cGMP and NTproBNP levels in healthy controls and HF patients at admission and pre-discharge.

4.4 Outcomes

Out of the 336 patients admitted with acute HF recruited, 319 were discharged and 17 died as inpatients. The median duration of hospital inpatient stay for HF patients who were discharged was 11 [range 0 to 104] days. All patients were followed up for 180 days with no patients lost to follow up. The outcomes of the HF patients are summarised in Table 4.3.

Table 4.3 Outcomes for acute HF patients

	n = 336
Dead	63 (18.8%)
HF readmission	45 (13.4%)
Dead or HF readmission	101 (30.0%)
ACS	7 (2.1%)
Revascularisation (PCI or CABG)	4 (1.2%)
Valve replacement	15 (4.5%)
CRT placement	11 (3.3%)
ICD placement	4 (1.2%)

4.5 Admission peptide levels

4.5.1 Linear analysis

ProUGN

Univariate comparison of admission ProUGN levels by demographic factors and outcomes are shown in Table 4.4 and Figures 4.3, 4.4 and 4.5.

Admission ProUGN levels were not significantly greater in those that died compared to survivors at the end of follow up. Admission ProUGN was significantly inversely correlated with eGFR but showed no strong associations with age or EF (Figure 4.6). ProUGN was also not significantly associated with admission sodium (r=.019, p=.726), LVIDS (r=-.01, p=.909) or mean E/é (r=.01, p=.927), plots not shown.

Table 4.4 Univariate analysis of admission ProUGN in HF patients

	Admission ProUGN[a] (pmol·l^{-1})	**p[b]**
Males *vs* females	1255 (890 to 1750) *vs* 1428 (937 to 1799)	.053
Past medical history *vs* none		
IHD	1257 (929 to 1732) *vs* 1322 (894 to 1807)	.953
Hypertension	1373 (921 to 1758) *vs* 1208 (869 to 1766)	.219
Diabetes	1346 (999 to 1823) *vs* 1257 (858 to 1751)	.200
AF	1263 (901 to 1757) *vs* 1324 (904 to 1766)	.714
Admission medications *vs* none		
Aspirin	1345 (998 to 1782) *vs* 1247 (805 to 1751)	.096
Diuretic	1297 (930 to 1766) *vs* 1286 (829 to 1737)	.407
Betablocker	1304 (914 to 1737) *vs* 1265 (842 to 1831)	.567
ACEi or ARB	1355 (941 to 1772) *vs* 1191 (809 to 1745)	.142
CCB	1370 (1028 to 1744) *vs* 1221 (871 to 1773)	.256
ARA	942 (694 to 1587) *vs* 1322 (929 to 1795)	.102
Statin	1373 (973 to 1748) *vs* 1208 (769 to 1830)	.156
Nitrate	1330 (884 to 1801) *vs* 1293 (919 to 1760)	.934
Outcomes *vs* none		
Death	1462 (942 to 2024) *vs* 1223 (896 to 1735)	.124
HF readmission	1304 (928 to 1609) *vs* 1213 (901 to 1756)	.836
Death or HF readmission	1370 (928 to 1835) *vs* 1223 (901 to 1758)	.469

[a] Median (IQR)
[b] t test of log values

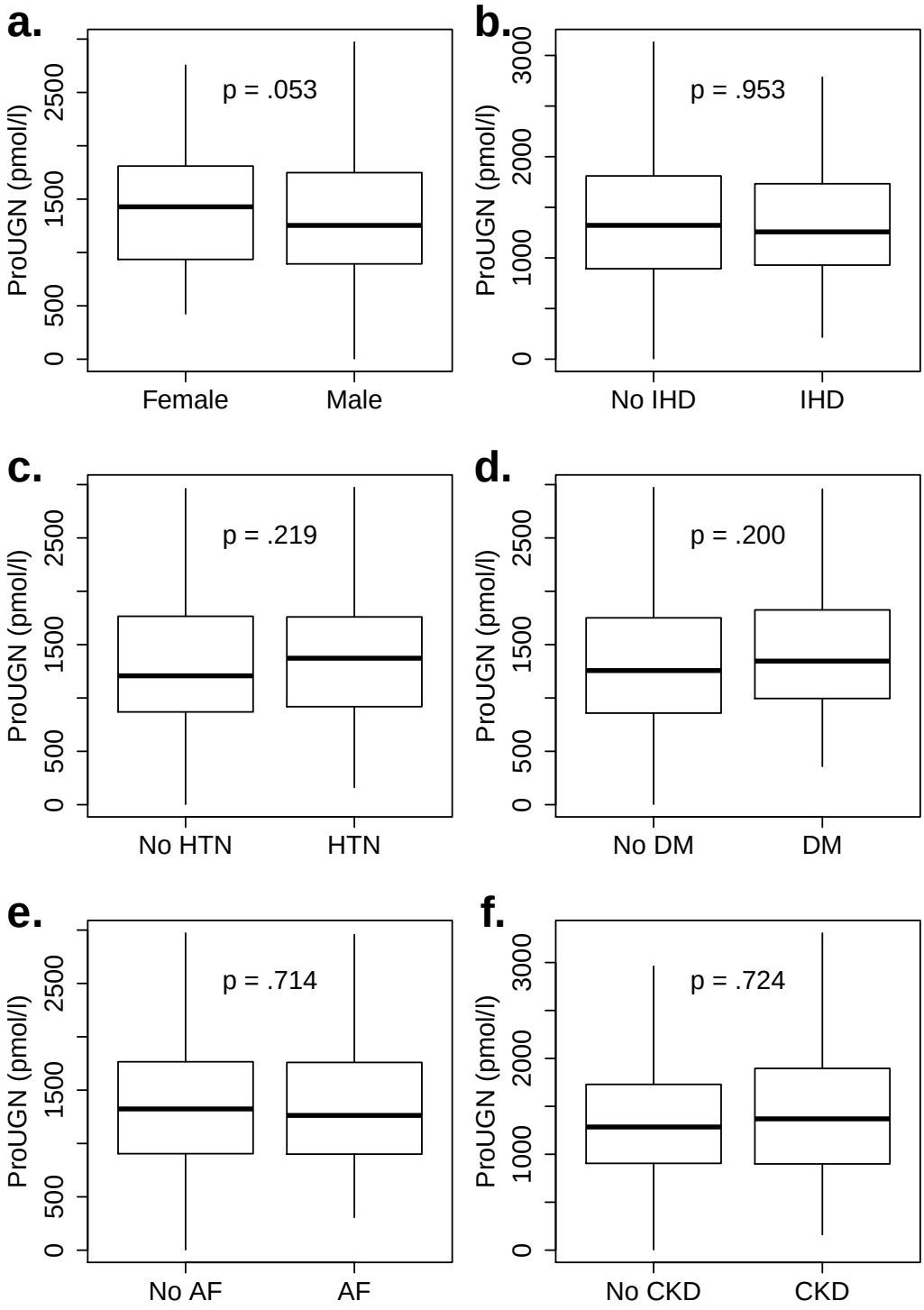

Figure 4.3 Admission ProUGN levels by demographics and past medical history. ProUGN levels were not significantly different for any of the factors. p values are shown for the t test.

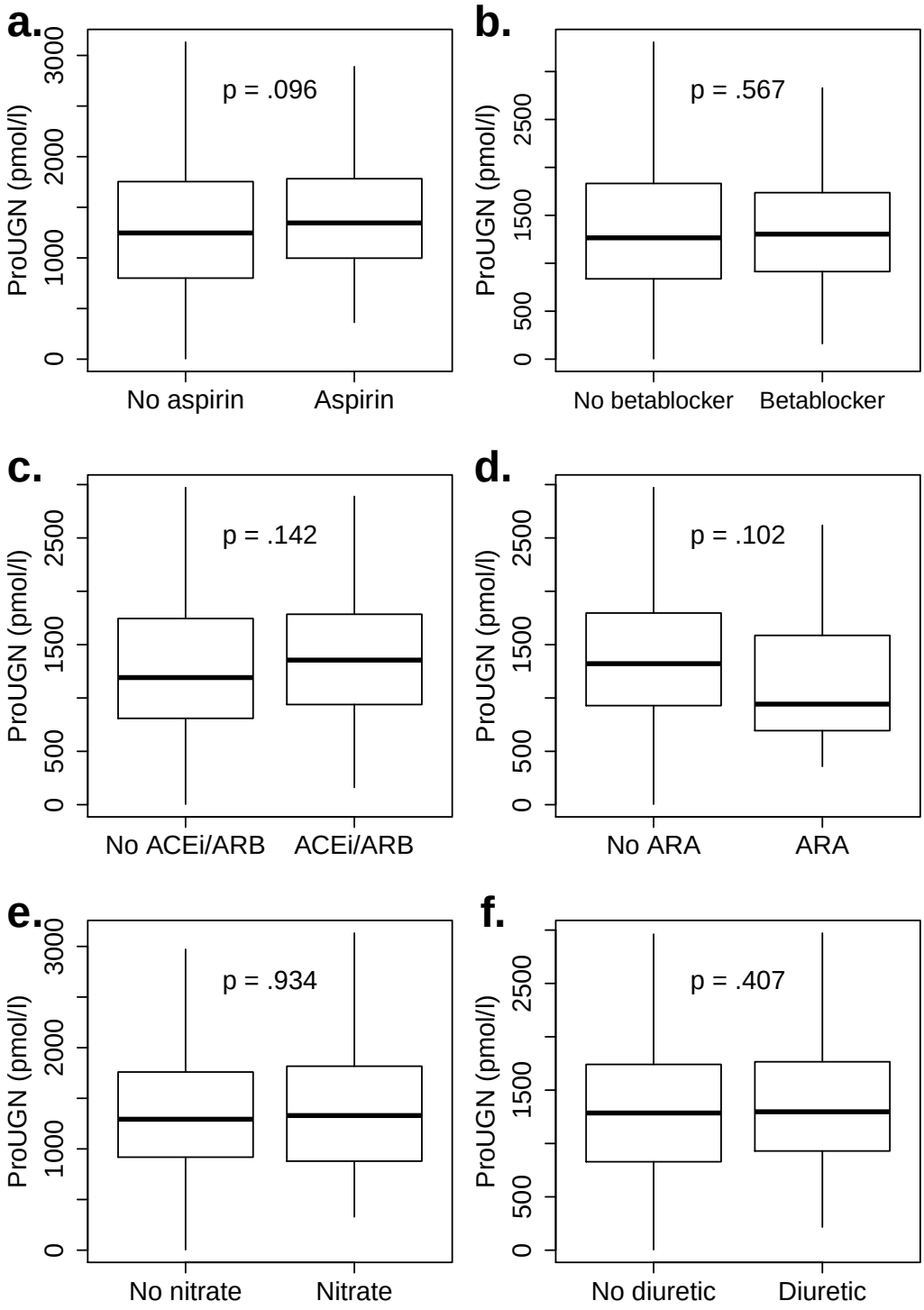

Figure 4.4 Admission ProUGN levels by medications on admission. ProUGN levels were not significantly different for any of the factors. p values are shown for the t test.

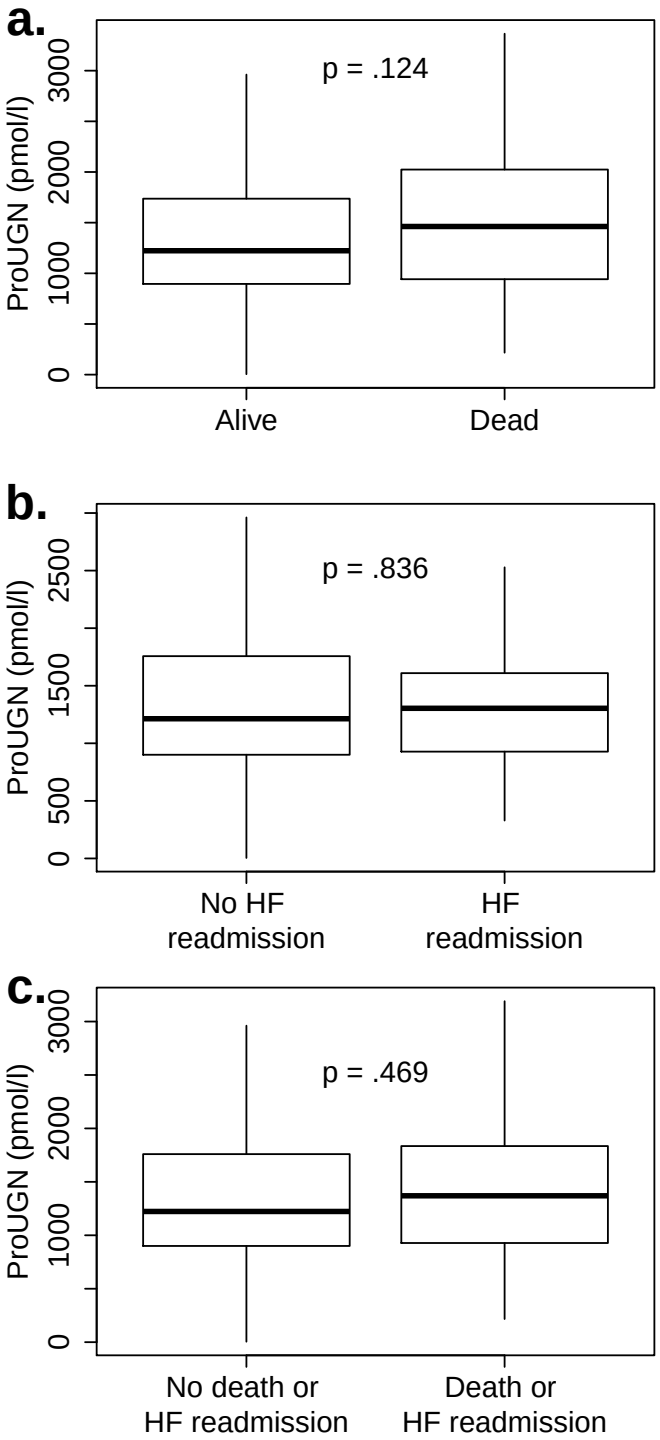

Figure 4.5 Admission ProUGN levels by outcome. ProUGN levels were not significantly different in those with any of the outcomes compared to those without. p values are shown for the t test.

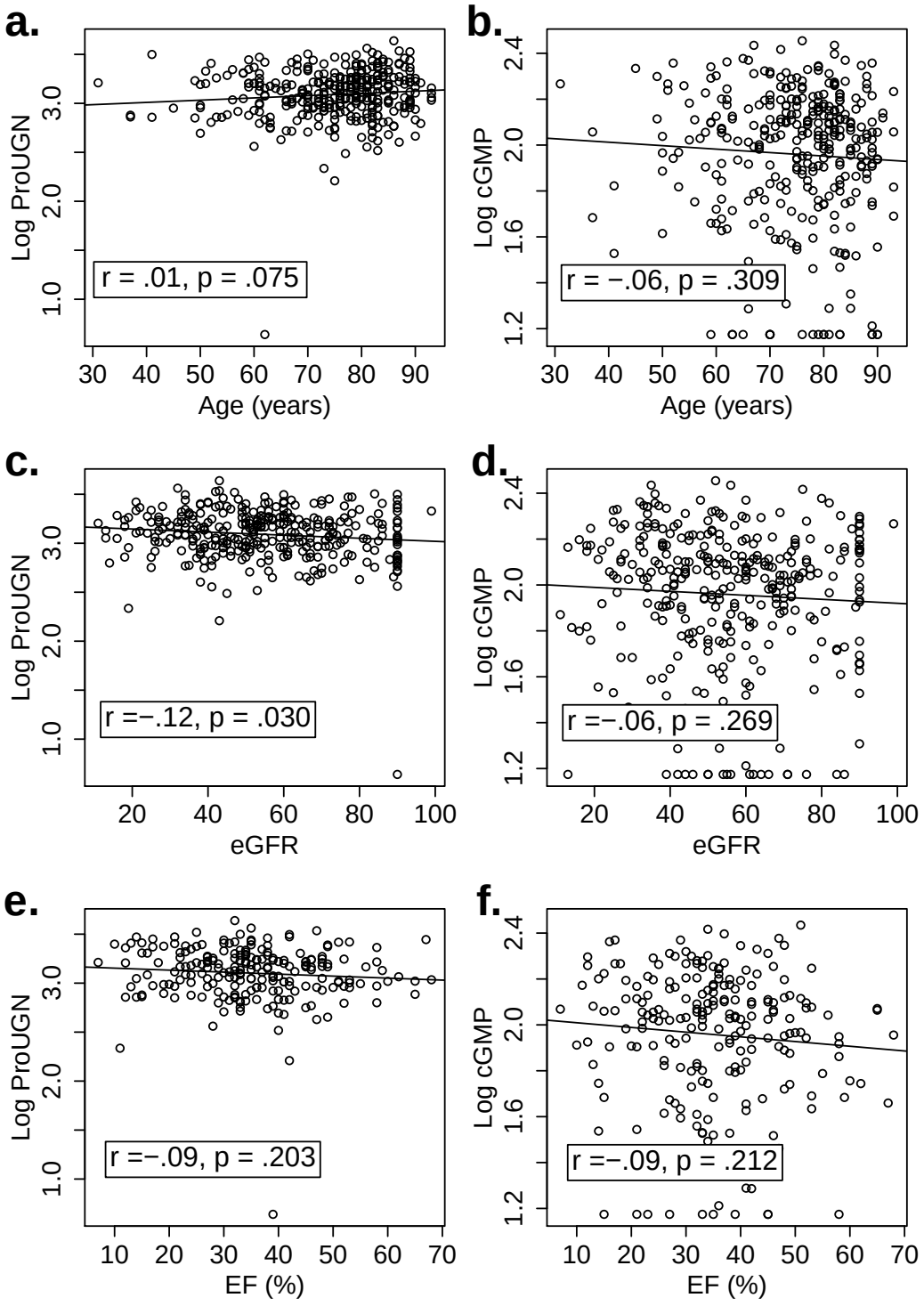

Figure 4.6 Scatter plots of ProUGN and cGMP at admission. ProUGN was significantly inversely correlated with eGFR. Pearson's r coefficient is shown in the boxes.

cGMP

Comparison of admission cGMP levels by demographic factors and outcomes are shown in Table 4.5 and Figures 4.7, 4.8 and 4.9.

The were no significant differences in cGMP levels at admission with demographic factors, medications and outcomes, although levels were greater but not significantly so in those taking nitrates compared to those not (116 [87 to 152] vs 108 [58 to 147] nmol·l^{-1}, p=.051) respectively, which accords with their mechanism of action. Admission cGMP was not significantly correlated with age, eGFR or EF (shown in Figure 4.6) or admission sodium (r=-.04, p=.496), LVIDS (r=.12, p=.116) or mean E/é (r=.06, p=.557), plots not shown.

Table 4.5 Univariate analysis of admission cGMP in HF patients

	Admission cGMP[a] (nmol·l^{-1})	**p[b]**
Males *vs* females	110 (63 to 147) *vs* 107 (80 to 153)	.311
Past medical history *vs* none		
IHD	107 (58 to 141) *vs* 111 (68 to 149)	.160
Hypertension	114 (67 to 153) *vs* 98 (65 to 134)	.098
Diabetes	115 (55 to 151) *vs* 107 (73 to 145)	.518
AF	109 (66 to 149) *vs* 109 (66 to 147)	.661
Admission medications *vs* none		
Aspirin	111 (60 to 147) *vs* 108 (68 to 149)	.862
Diuretic	114 (70 to 152) *vs* 106 (63 to 140)	.530
Betablocker	113 (67 to 147) *vs* 105 (65 to 149)	.586
ACEi or ARB	111 (63 to 156) *vs* 106 (68 to 139)	.931
CCB	107 (54 to 140) *vs* 110 (68 to 149)	.455
ARA	114 (86 to 163) *vs* 109 (66 to 146)	.403
Statin	111 (66 to 149) *vs* 107 (66 to 144)	.874
Nitrate	116 (87 to 152) *vs* 108 (58 to 147)	.051
Outcomes *vs* none		
Death	97 (56 to 126) *vs* 111 (66 to 149)	.160
HF readmission	109 (81 to 150) *vs* 107 (64 to 147)	.697
Death or HF readmission	107 (70 to 136) *vs* 111 (66 to 149)	.225

[a] Median (IQR)
[b] t test of log values

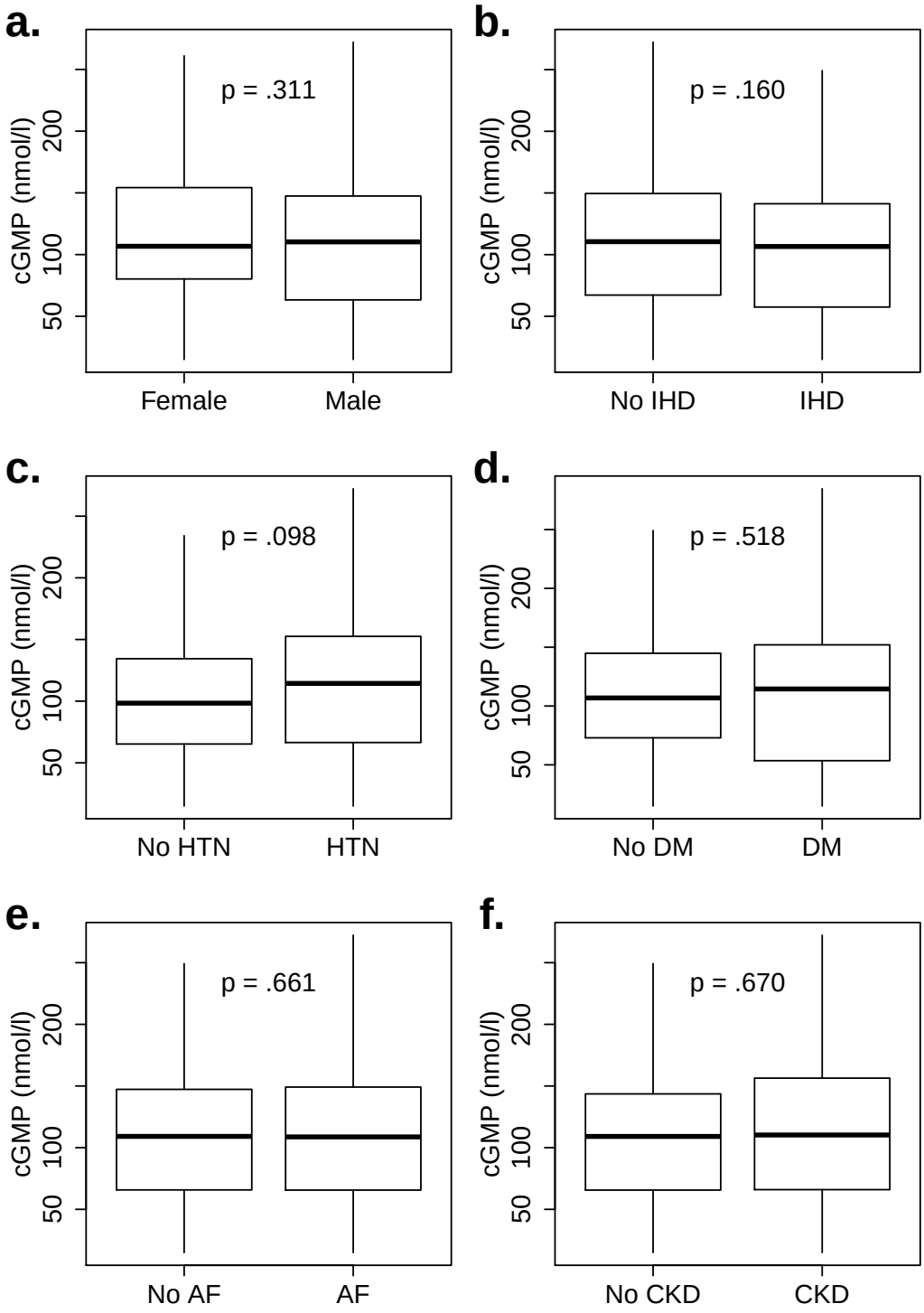

Figure 4.7 Admission cGMP levels by demographics and past medical history. cGMP levels were not significantly different for any of the factors. p values are shown for the t test.

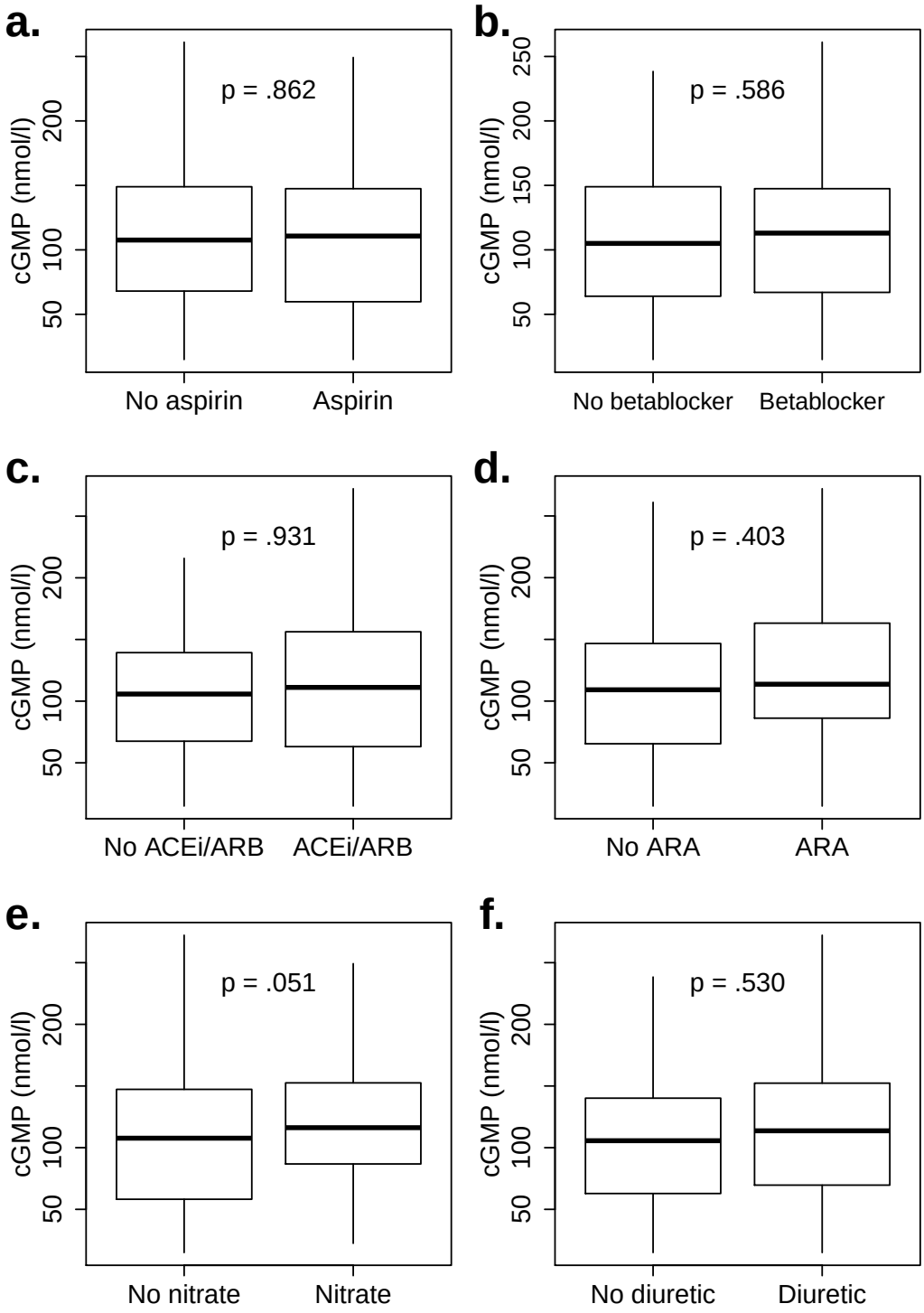

Figure 4.8 Admission cGMP levels by medications on admission. cGMP levels were not significantly different in patients taking these medications compared to those not. p values are shown for the t test.

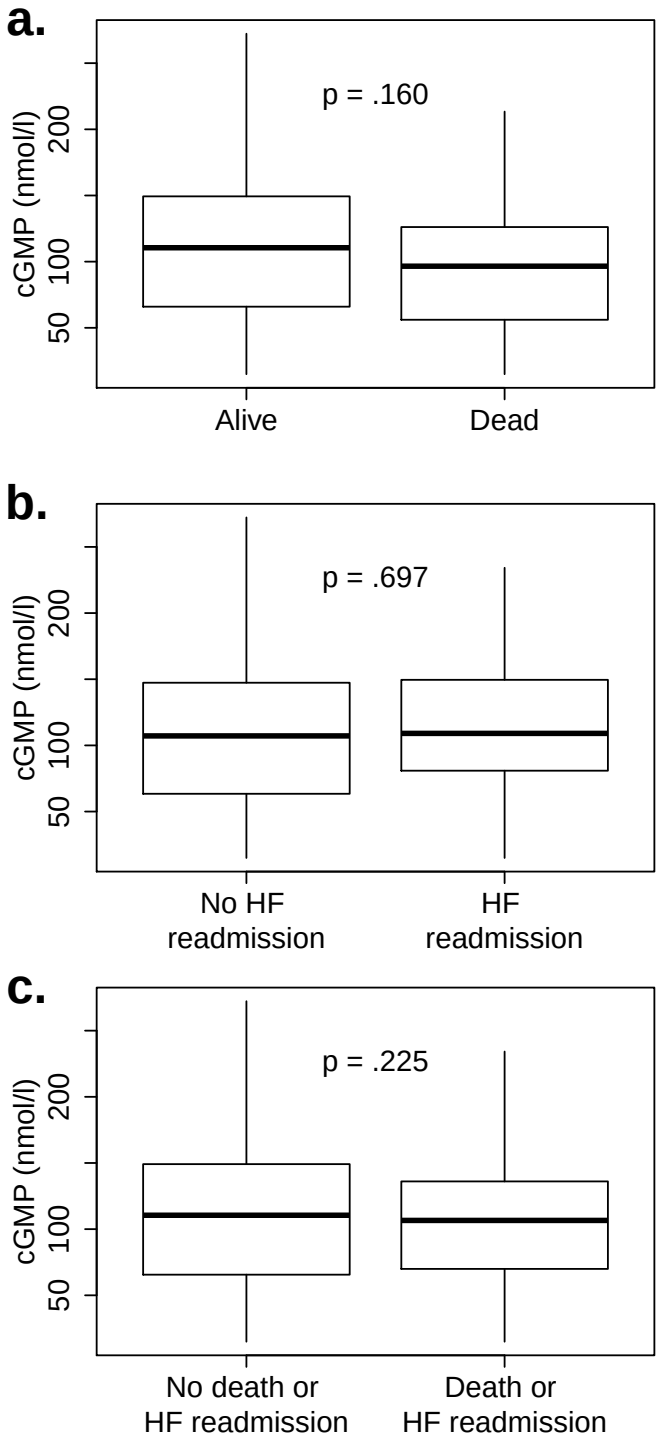

Figure 4.9 Admission cGMP levels by outcome. cGMP levels were not significantly different in those with any of the outcomes compared to those without. p values are shown for the t test.

4.5.2 Logistic regression

Odds ratios (OR) with 95% C.I.s for variables predictive for the outcomes of all cause mortality, readmission with HF and all cause mortality or HF readmission are shown using forest plots in Figure 4.10.

Age (OR=1.06 [1.03 to 1.10], p<.001), NTproBNP (OR=5.07 [2.40 to 11.7], p<.001) and a past history of IHD (OR=1.87 [1.08 to 3.27], p=.026) were all associated with increased risk of all cause mortality while a higher eGFR (OR=0.97 [0.96 to 0.99], p<.001), taking a betablocker (OR=0.39 [0.22 to 0.67], p=.001), ACEi or ARB (OR=0.27 [0.15 to 0.47], p<.001) and diuretic (OR=0.25 [0.14 to 0.47], p<.001) at discharge were associated with a significantly reduced risk of this outcome in univariate analysis (Figure 4.10a). In multivariate analysis age (OR=1.04 [1.00 to 1.05], p=.049), NTproBNP (OR=2.72 [1.26 to 6.49], p=.017) remained significant independent predictors of a worse outcome while ACEi/ARB use (OR=0.43 [0.20 to 0.93], p=.031) was associated with a better outcome (Figure 4.10b).

No variables were significantly associated with an increased risk of readmission with HF in univariate analysis (Figure 4.10c).

Age (OR=1.03 [1.00 to 1.05], p=.030) and NTproBNP (OR=2.77 [1.61 to 5.03], p<.001) were both associated with increased risk of the outcome all cause mortality or HF readmission while a higher GFR (OR=0.98 [0.97 to 0.99], p=.002), taking betablockers (OR=0.42 [0.26 to 0.68], p<.001), an ACEi or ARB (OR=0.42 [0.26 to 0.68], p<.001) and diuretics (OR=0.39 [0.22 to 0.69], p<.001 at discharge were associated with a reduced risk of this outcome in univariate analysis (Figure 4.10d). In multivariate analysis only a higher NTproBNP (OR=2.69 [1.47 to 5.25], p=.002) was associated with increased risk of the combined endpoint while taking a betablocker at discharge was associated with a reduced risk of the outcome (OR=0.54 [0.30 to 0.97], p=.038) (Figure 4.10e).

A greater admission ProUGN/cGMP ratio was significantly associated with increased risk of all cause mortality (OR=2.09 [1.04 to 4.25], p=.039) but not HF readmission (OR=0.84 [0.38 to 1.90], p=.663) or the combined endpoint (OR=1.52 [0.84 to 2.78], p=.167) in univariate analysis. The admission NT-

proBNP/cGMP ratio was a significant predictor of all cause mortality (OR=3.33 [1.93 to 6.02], p<.001) and all cause mortality or HF readmission (OR=2.31 [1.49 to 3.69], p<.001) but not HF readmission alone (OR=1.45 [0.85 to 2.63], p=.196) in univariate analysis.

4.5.3 Cox hazards regression

Hazard ratios (HR) with 95% C.I.s for variables predictive for the endpoints of time to all cause mortality, time to readmission with HF and time to all cause mortality or HF readmission are shown using forest plots in Figure 4.11.

Age (HR=1.06 [1.03 to 1.09], p<.001), NTproBNP (HR=4.95 [2.36 to 10.4], p<.001) and past history of IHD (HR=1.72 [1.05 to 2.82], p=.032) were associated with a greater risk of earlier all cause mortality, while a higher eGFR (HR=0.97 [0.96 to 0.99], p<.001), taking betablockers (HR=0.40 [0.24 to 0.66], p<.001), ACEi or ARBs (HR=0.29 [0.18 to 0.49], p<.001) and diuretics (HR=0.28 [0.17 to 0.46], p<.001) at discharge were associated with a reduced risk or early all cause mortality in univariate analysis (Figure 4.11a).

In multivariate analysis, age (HR=1.03 [1.00 to 1.06], p=.023) and NT-proBNP (HR=2.85 [1.35 to 5.98], p=.006) were independently associated with increased risk of early all cause mortality, while taking and ACEi or ARB (HR=0.50 [0.26 to 0.94], p=.044) and diuretic use (HR=0.45 [0.26 to 0.76], p=.003) at discharge was associated with a reduced risk (Figure 4.11b).

No variables were significantly associated with risk of early HF readmission in univariate analysis (Figure 4.11c).

Age (HR=1.02 [1.00 to 1.04], p=.022) and NTproBNP (HR=2.60 [1.58 to 4.29], p<.001) were significantly associated with increased risk of early all cause mortality or HF readmission while a higher eGFR (HR=0.98 [0.97 to 0.99], p=.001), taking betablockers (HR=0.46 [0.31 to 0.69], p<.001), ACEi or ARBs (HR=0.46 [0.31 to 0.68], p<.001) and diuretics (HR=0.41 [0.27 to 0.63], p<.001) at discharge were associated with a reduced risk of this early outcome in univariate analysis (Figure 4.11d). In multivariate analysis, NTproBNP (HR=2.55 [1.48 to 4.39], p<.001) remained associated with increased risk of early all cause mortality or HF readmission while betablocker use (HR=0.58

[0.37 to 0.92], p=.019) was associated with a reduced risk of the early combined endpoint (Figure 4.11e).

The admission ProUGN/cGMP ratio was significantly associated with early all cause mortality (HR=1.99 [1.07 to 3.72], p=.030) but not early readmission with HF (HR=0.83 [0.41 to 1.69], p=.609) or the combined endpoint (HR=1.41 [0.86 to 2.32], p=.178) in univariate analysis. The admission NTproBNP/cGMP ratio was significantly associated with early all cause mortality (HR=3.03 (1.87 to 4.90), p<.001), early all cause mortality or HF readmission (HR=2.08 [1.44 to 3.01], p<.002) but not early HF readmission alone (HR=1.40 [0.84 to 2.33], p=.193) in univariate analysis.

4.5.4 Kaplan-Meier analysis

Kaplan-Meier plots stratified by median admission NTproBNP, ProUGN and cGMP level for outcomes of all cause mortality, HF readmission and all cause mortality or HF readmission are shown in Figures 4.12, 4.13, and 4.14. HF patients were stratified by median cGMP level in order to assess whether greater or lower admission cGMP levels were associated with better or worse prognosis.

Higher levels of NTproBNP were significantly associated with worse outcomes of all cause mortality and all cause mortality or HF readmission. ProUGN and cGMP levels at admission were not associated with any adverse outcome.

Admission ProUGN/cGMP ratio was not strongly associated with any adverse outcome, while a higher NTproBNP/cGMP ratio was significantly associated with earlier all cause mortality and all cause mortality or HF readmission (Figures 4.15 and 4.16).

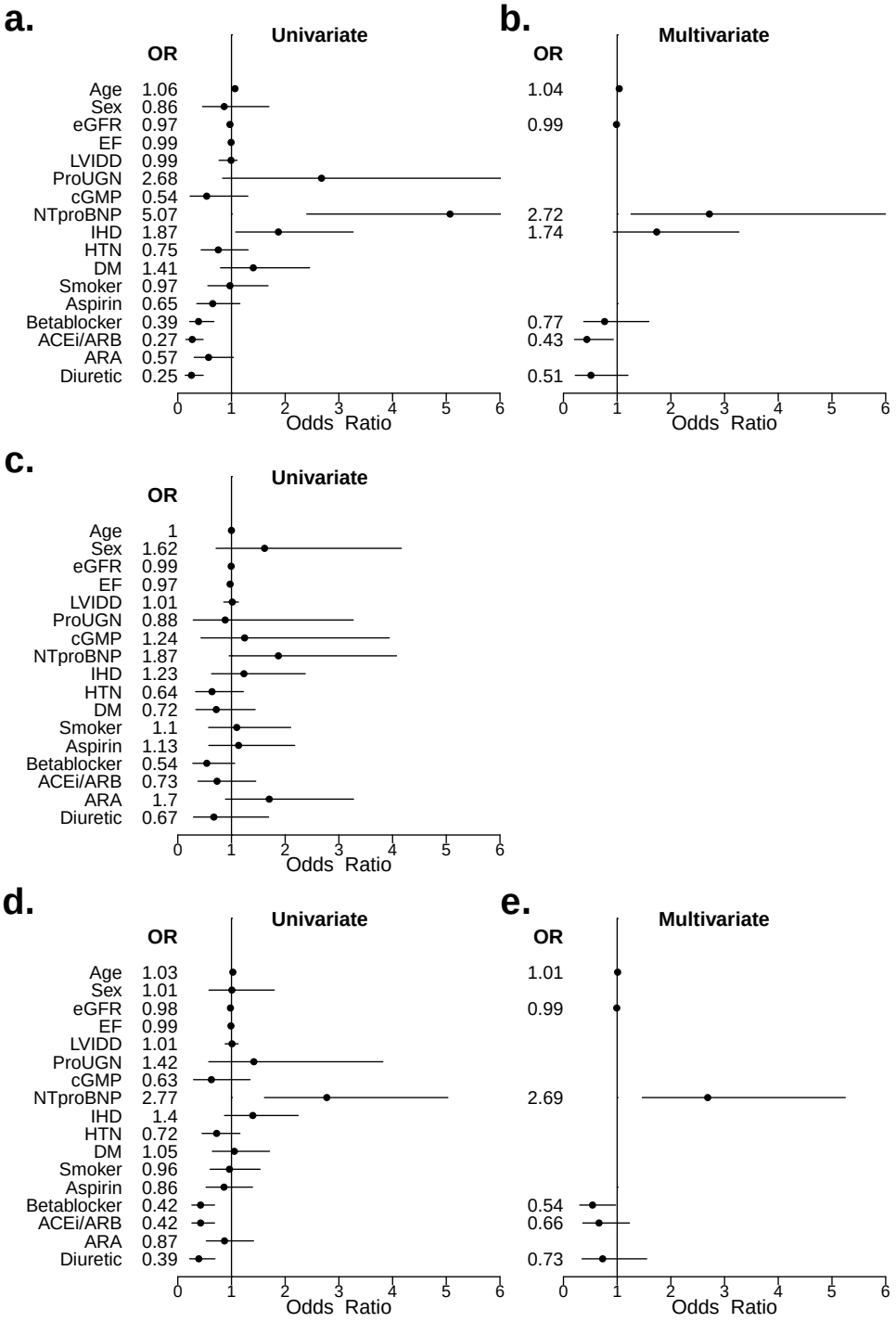

Figure 4.10 Forest plots showing odds ratios with 95% C.I.s for logistic regression analysis of admission ProUGN, NTproBNP and cGMP for outcomes: All cause mortality (**a** & **b**), HF readmission (**c**) and all cause mortality or HF readmission (**d** & **e**). Admission eGFR and medications on discharge are used.

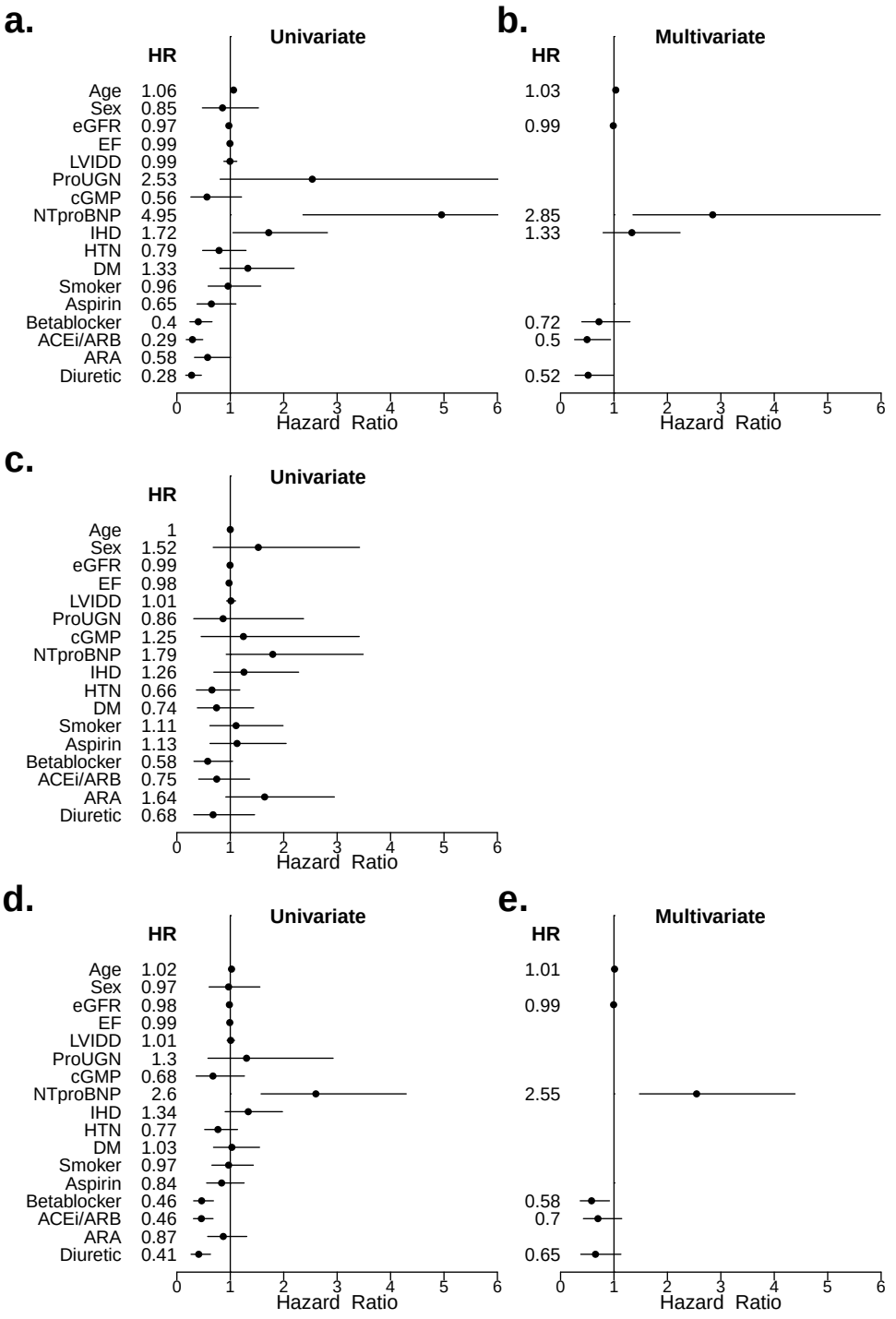

Figure 4.11 Forest plots showing hazard ratios with 95% C.I.s for Cox hazards regression analysis of admission ProUGN, NTproBNP and cGMP for outcomes: All cause mortality (**a** & **b**), HF readmission (**c**) and all cause mortality or HF readmission (**d** & **e**). Admission eGFR and medications on discharge are used.

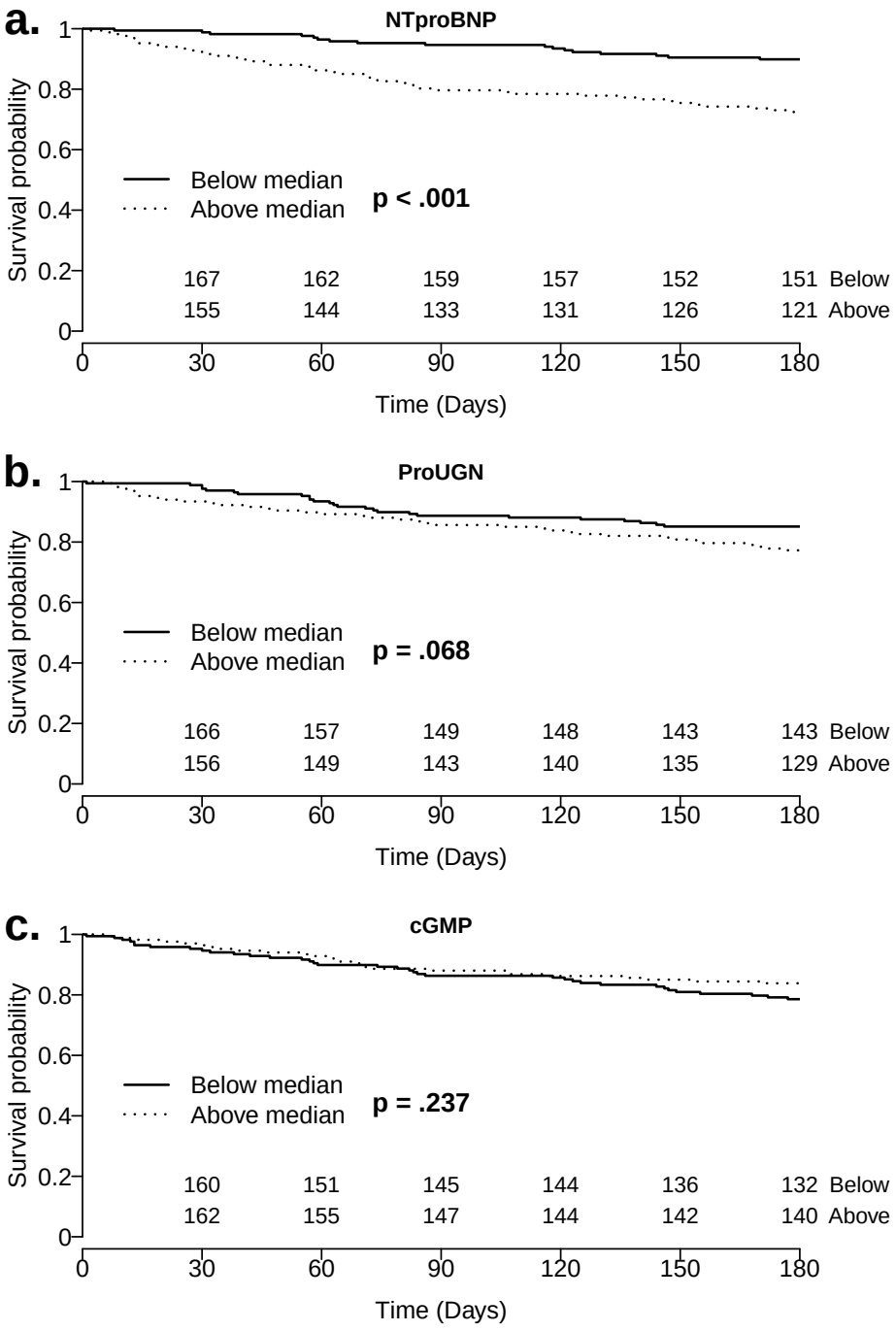

Figure 4.12 Kaplan-Meier plots for outcome all cause mortality stratified by admission **a.** NTproBNP **b.** ProUGN **c.** cGMP. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

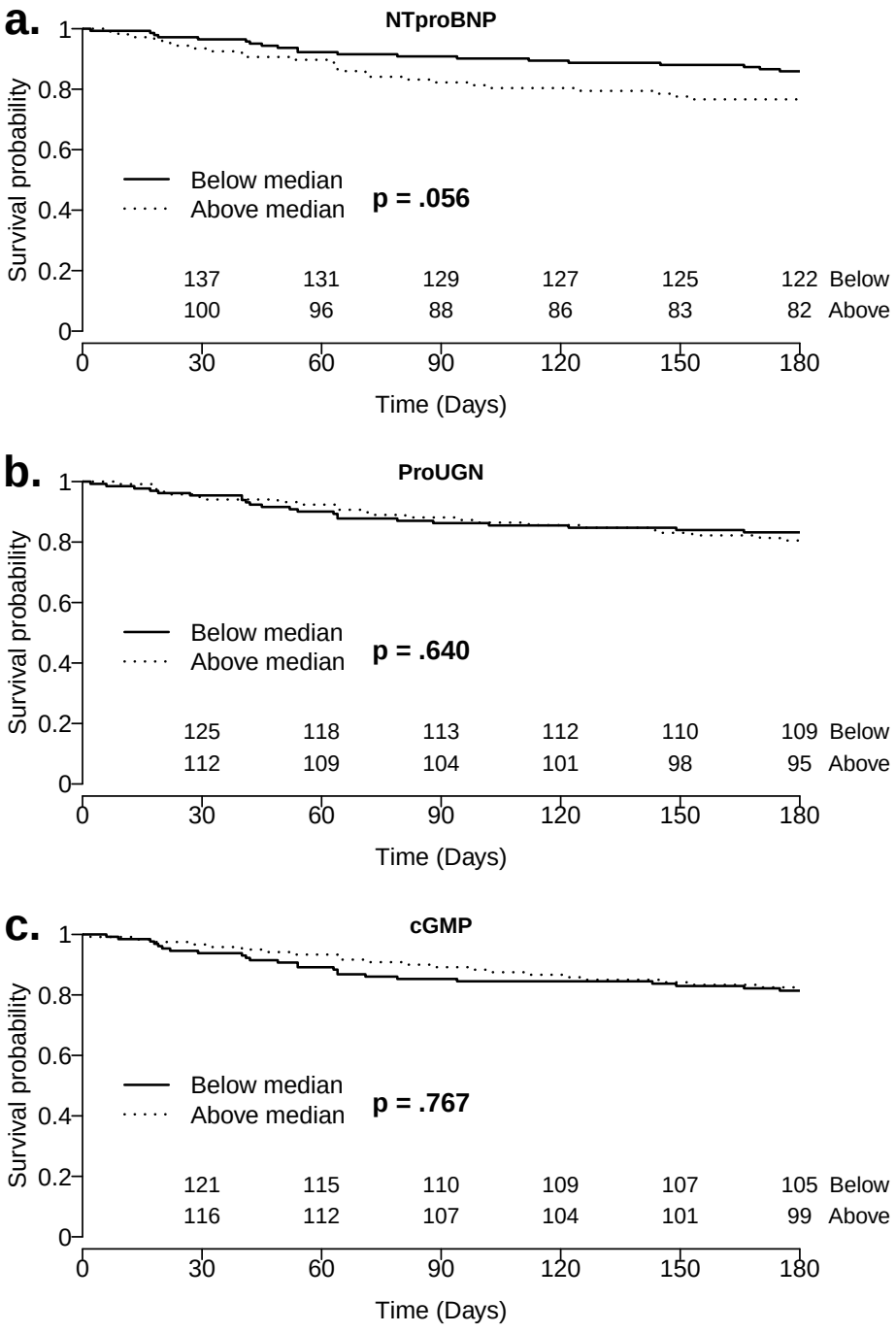

Figure 4.13 Kaplan-Meier plots for outcome of HF readmission stratified by admission **a.** NTproBNP **b.** ProUGN **c.** cGMP. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

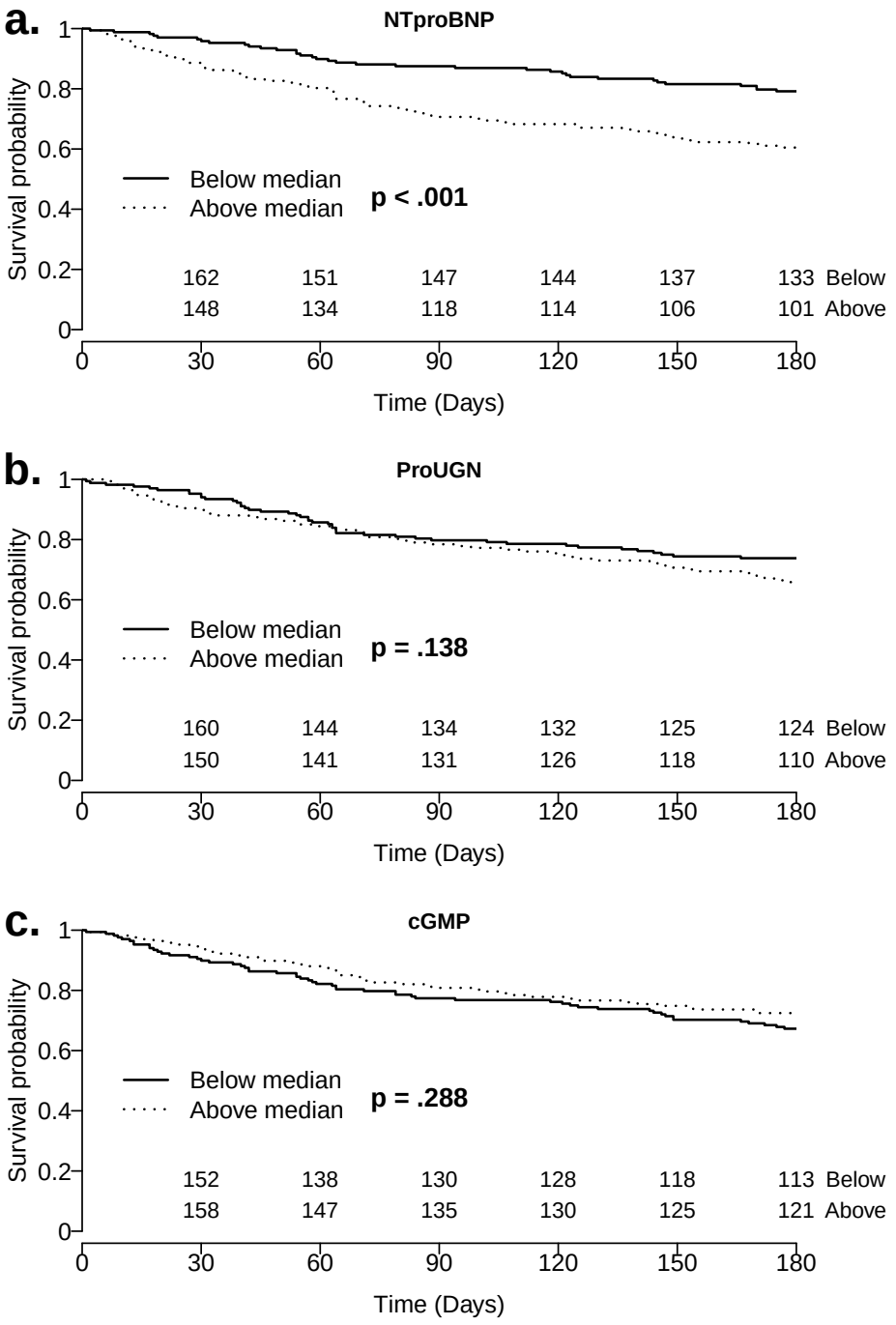

Figure 4.14 Kaplan-Meier plots for outcome all cause mortality or HF readmission stratified by admission **a.** NTproBNP **b.** ProUGN **c.** cGMP. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

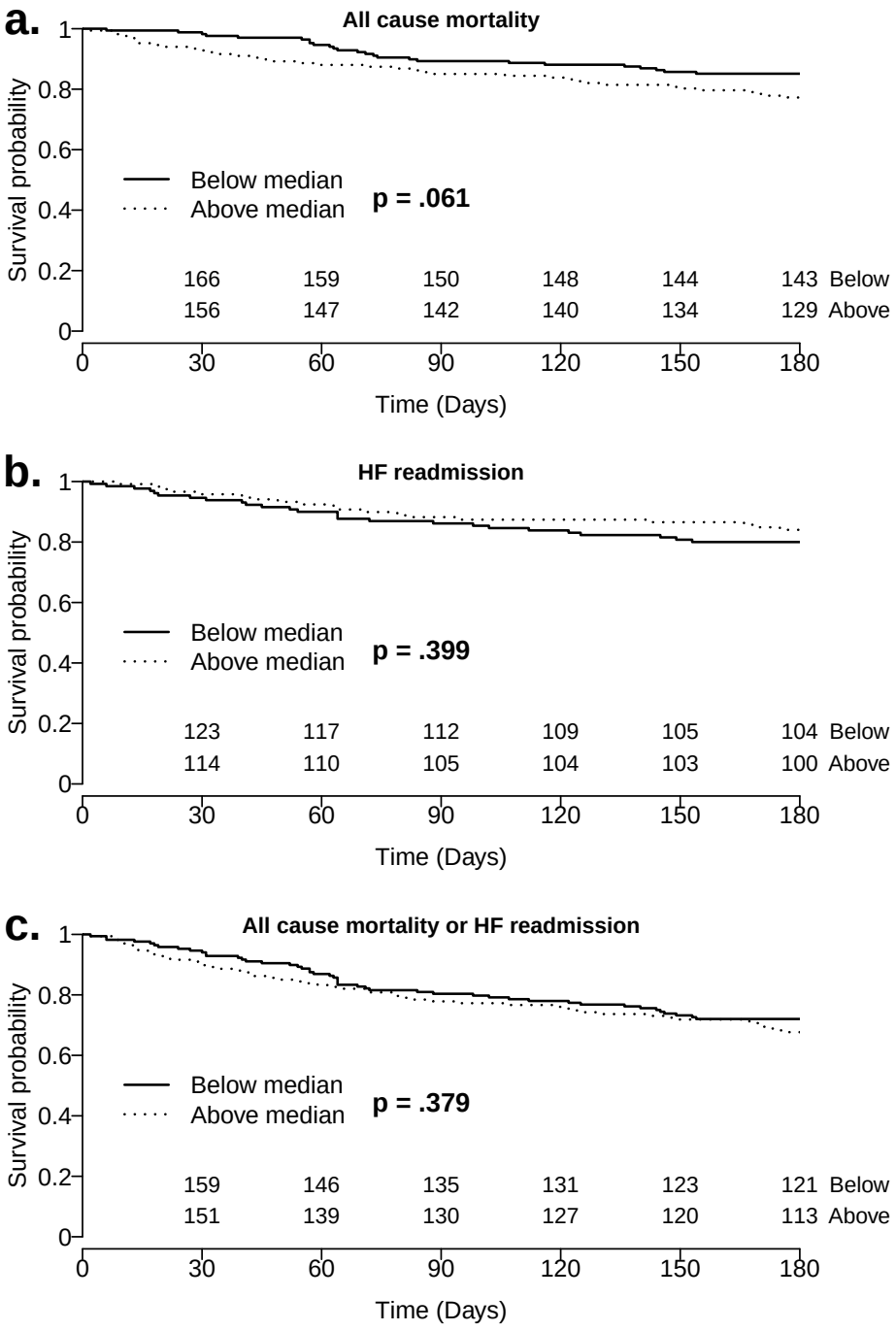

Figure 4.15 Kaplan-Meier plots stratified by median admission ProUGN/cGMP ratio for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

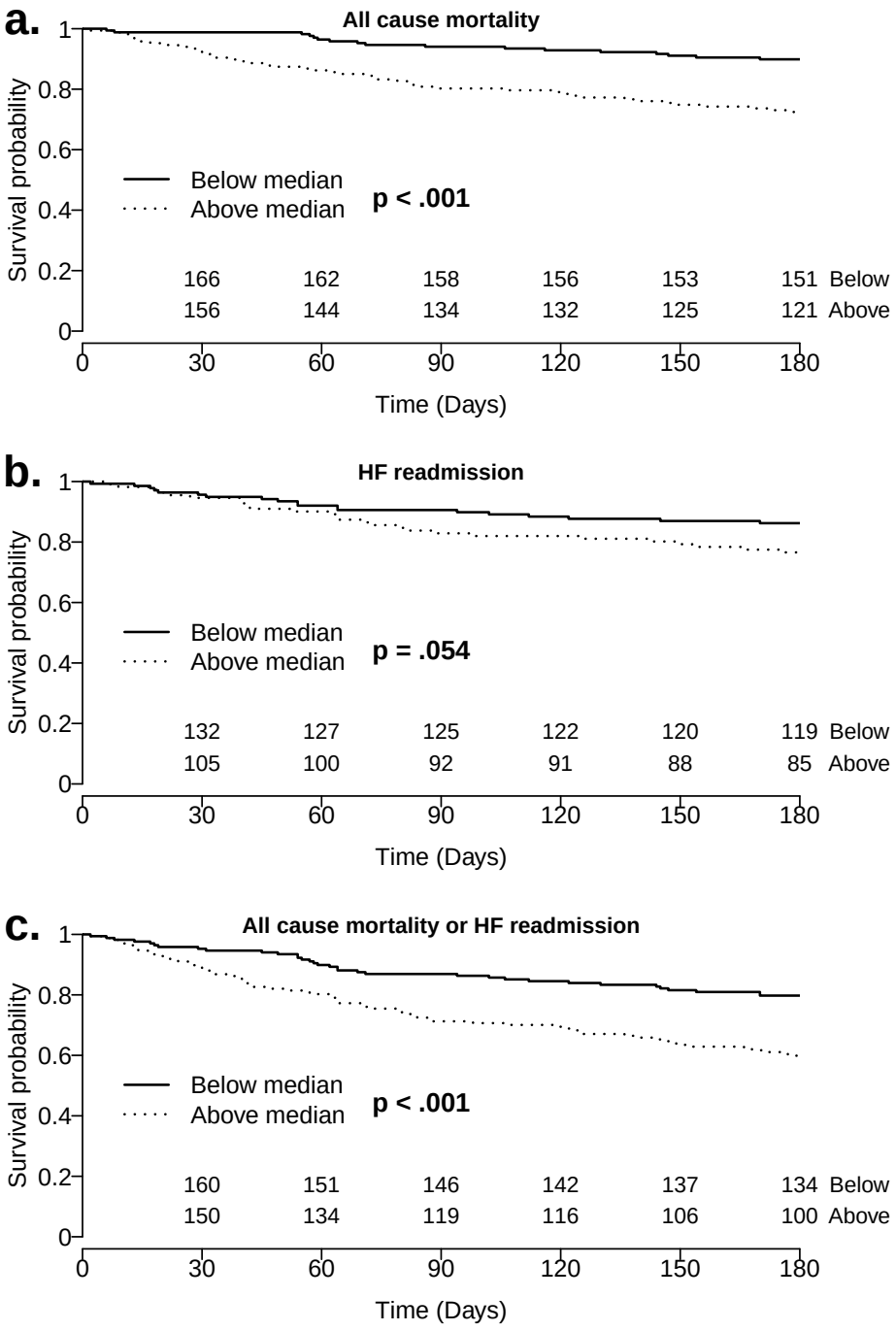

Figure 4.16 Kaplan-Meier plots stratified by median admission NTproBNP/cGMP ratio for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

4.5.5 ROC curve analysis

Classification by outcome for admission ProUGN, NTproBNP, cGMP as well as ProUGN/cGMP and NTproBNP/cGMP ratio is shown using ROC curves in Figure 4.17. For outcome all cause mortality, higher NTproBNP levels (AUC=.687 [.612 to .764]) showed significantly greater classification accuracy as measured by AUC compared to ProUGN (AUC=.568 [.487 to .650], p=.038) and cGMP (inverse AUC=.560 [.482 to .638], p=.026) in which lower levels were were associated with better outcomes (Figure 4.17a). For outcome HF readmission, NTproBNP (AUC=.581 [.489 to .612]) did not have significantly greater classification accuracy compared to ProUGN (inverse AUC=.517 [.458 to .607], p=.139) and cGMP (inverse AUC=.533 [.444 to .628], p=.086) (Figure 4.17b). NTproBNP (AUC=.638 [.573 to .704]) had significantly greater classification accuracy for the combined endpoint compared to ProUGN (AUC=.530 [.461 to .600], p=.022) and cGMP (inverse AUC=.525 [.458 to .593], p=.022) (Figure 4.17c). A higher NTproBNP/cGMP ratio had significantly greater classification accuracy compared to the ProUGN/cGMP ratio for endpoints all cause mortality (AUC=.680 [.601 to .754] vs AUC=.583 [.501 to .665], p=.026) and all cause mortality or HF readmission (AUC=.630 [.565 to .694] vs AUC=.544 [.475 .613], p=.018) but not HF readmission (AUC=.559 [.470 to .647] vs AUC=.522 [.430 to .613], p=.637).

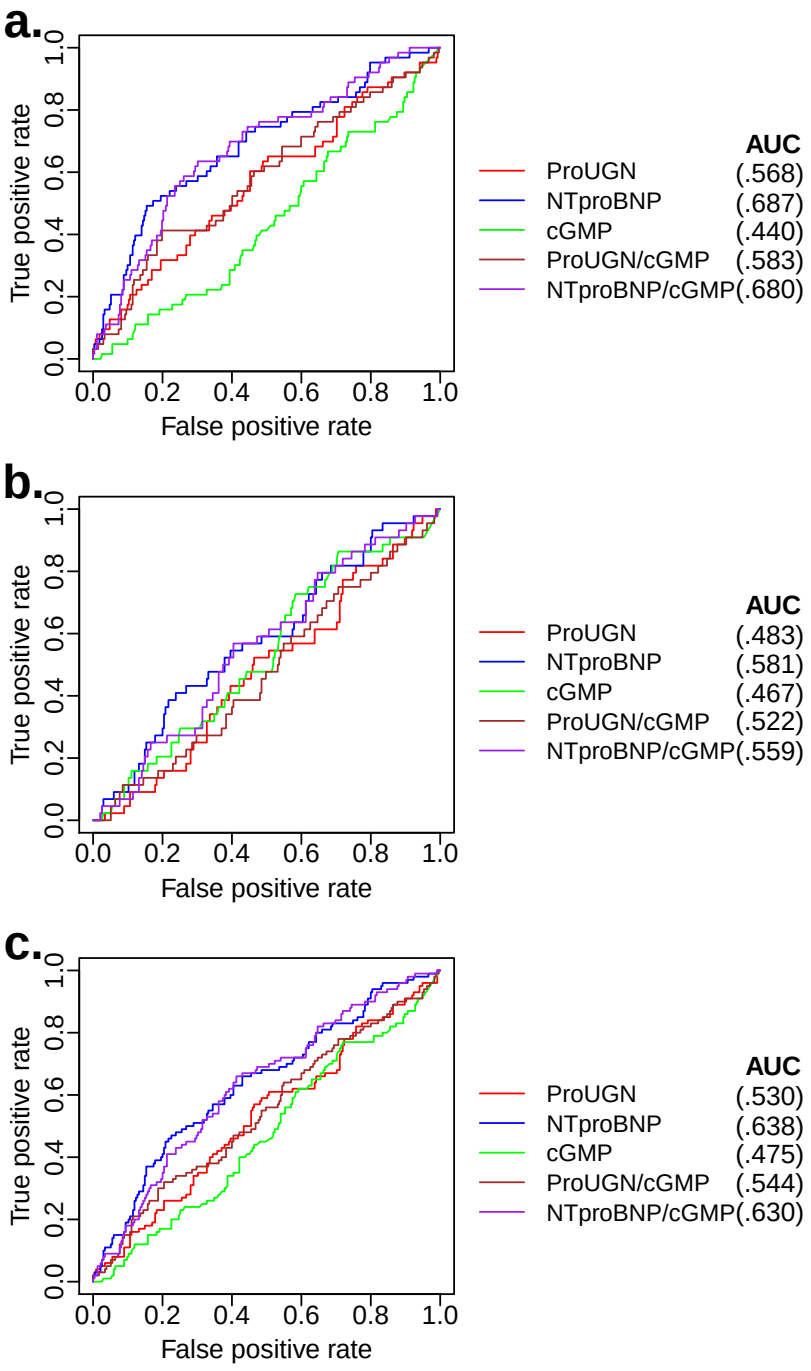

Figure 4.17 ROC curves for admission biomarkers for endpoints **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. AUC values are shown in brackets.

4.6 Pre-discharge peptide levels

4.6.1 Linear analysis

ProUGN

Univariate comparison of pre-discharge ProUGN levels by demographic factors and outcomes are shown in Table 4.6 and Figures 4.18, 4.19 and 4.20.

Pre-discharge ProUGN levels were significantly greater in those taking diuretics, betablockers and ACEi or ARBs. Pre-discharge ProUGN levels were significantly greater in those that died or had the combined endpoint of all cause mortality or HF readmission compared to those who did not. There were no strong associations with pre-discharge ProUGN and age, EF or eGFR (shown in Figure 4.21) and pre-discharge sodium (r=.04, p=.565), LVIDS (r=.14, p=.084) or mean E/é (r=.00, p=.990), plots not shown.

Table 4.6 Univariate analysis of pre-discharge ProUGN in HF patients

	Pre-discharge ProUGN[a] (pmol·l^{-1})	**p[b]**
Males *vs* females	4980 (3078 to 7592) *vs* 3918 (2821 to 6300)	.075
Past medical history *vs* none		
IHD	4818 (2983 to 7628) *vs* 4708 (2994 to 6948)	.383
Hypertension	4389 (2909 to 7337) *vs* 4862 (3591 to 7580)	.178
Diabetes	4122 (2943 to 6722) *vs* 4960 (3008 to 7644)	.376
AF	4477 (3355 to 6517) *vs* 4953 (2822 to 7709)	.925
Discharge medications *vs* none		
Aspirin	5073 (2991 to 7754) *vs* 4464 (2983 to 7220)	.474
Diuretic	4980 (3022 to 7409) *vs* 4033 (2276 to 5352)	.016
Betablocker	5149 (3115 to 8071) *vs* 4278 (2920 to 5742)	.021
ACEi or ARB	5095 (3184 to 7682) *vs* 4122 (2874 to 6140)	.035
CCB	5057 (2927 to 6654) *vs* 4723 (3009 to 7604)	.421
ARA	4862 (2927 to 7647) *vs* 4692 (3323 to 7026)	.851
Statin	4908 (3008 to 7463) *vs* 4708 (2964 to 6935)	.752
Nitrate	4558 (3008 to 8657) *vs* 4757 (2933 to 6922)	.181
Outcomes *vs* none		
Death	6218 (4114 to 8677) *vs* 4590 (2931 to 7026)	.048
HF readmission	5087 (3617 to 7644) *vs* 4723 (2909 to 6722)	.139
Death or HF readmission	4984 (3830 to 8318) *vs* 4517 (2845 to 6999)	.040

[a] Median (IQR)

[b] t test of log values

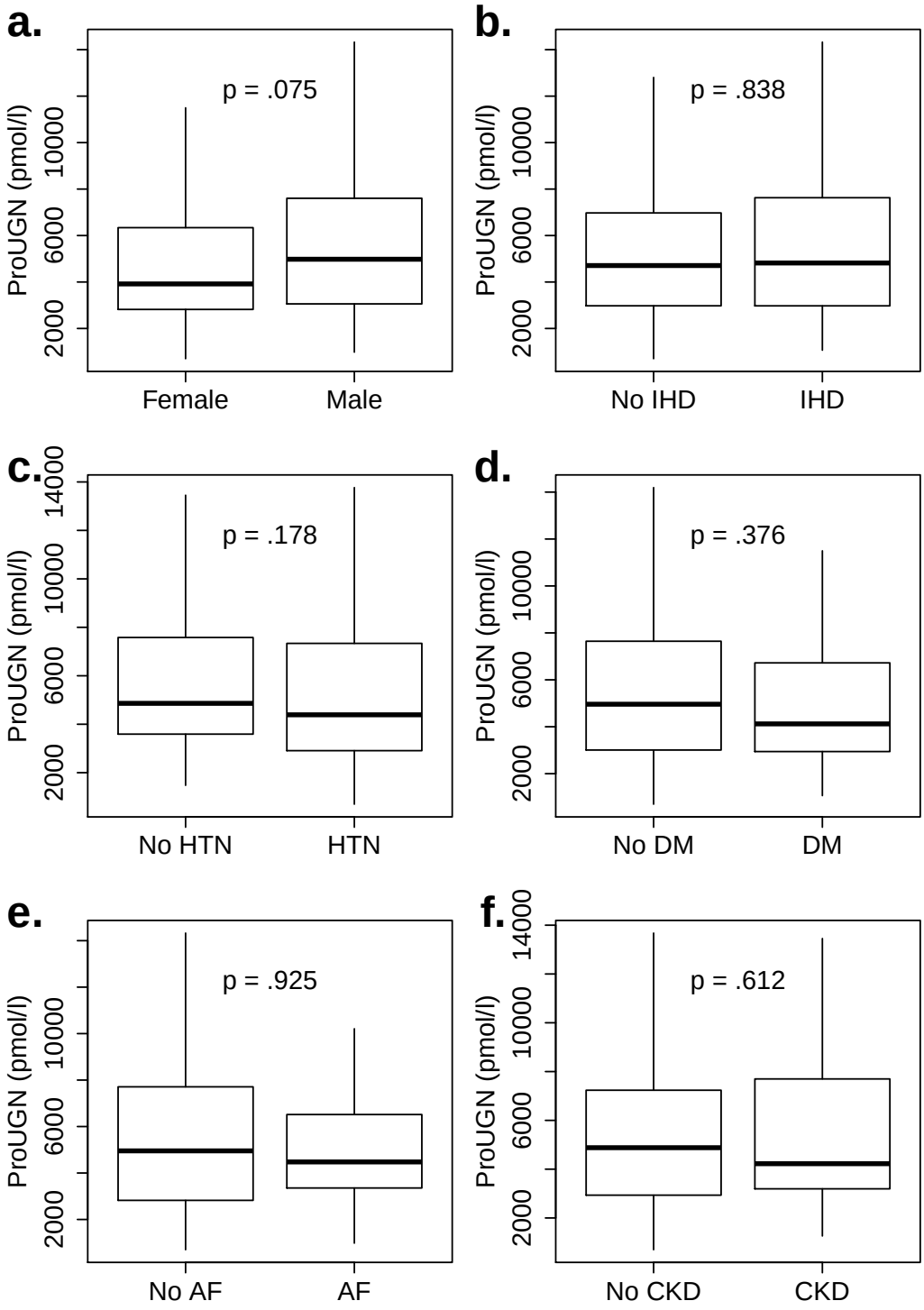

Figure 4.18 Pre-discharge ProUGN levels by demographics and past medical history. ProUGN levels were not significantly different for any of the factors p values are shown for the t test.

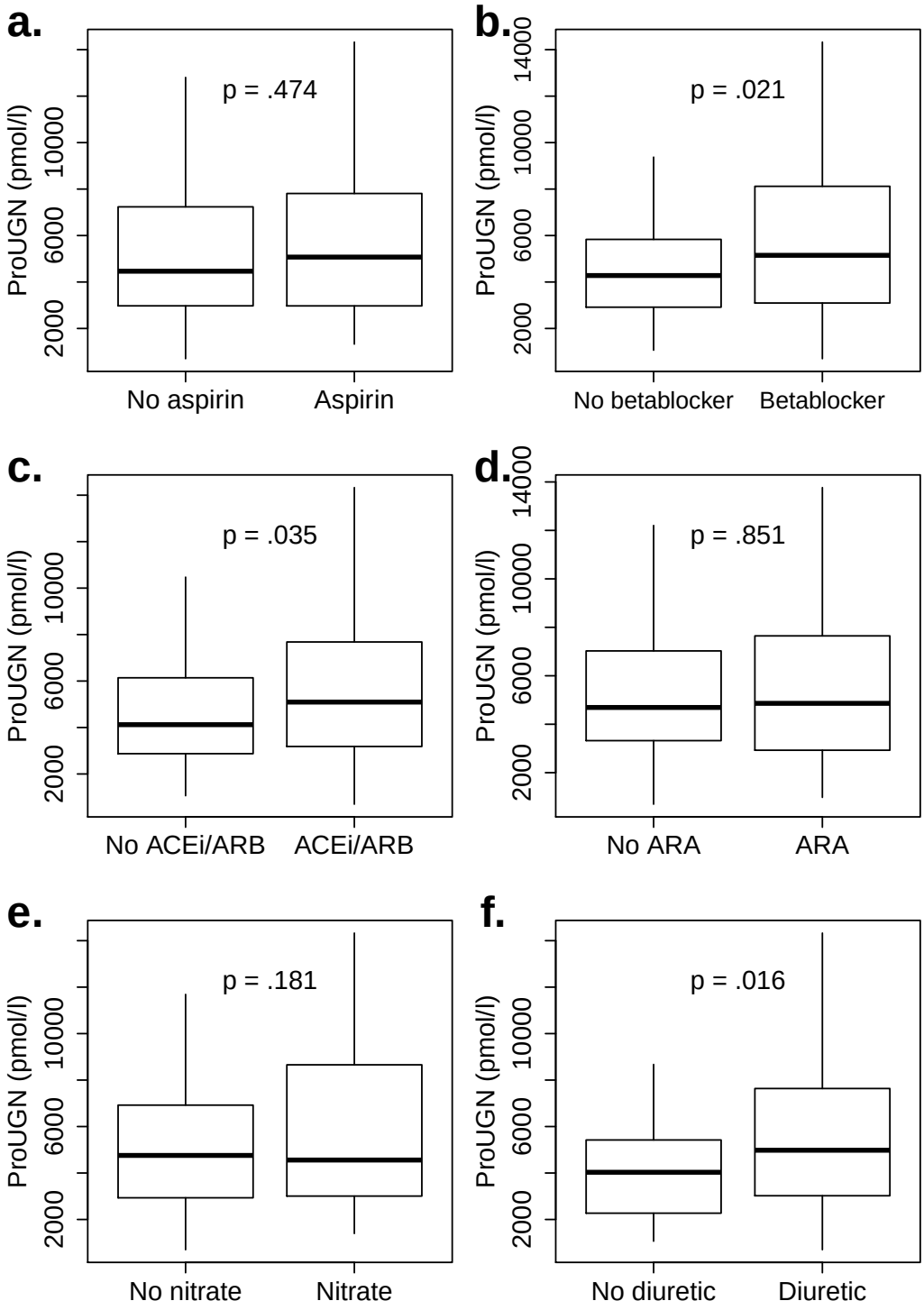

Figure 4.19 Pre-discharge ProUGN levels by medications at discharge. ProUGN levels were significantly greater in those taking betablockers, ACEi/ARBs, and diuretics. p values are shown for the t test.

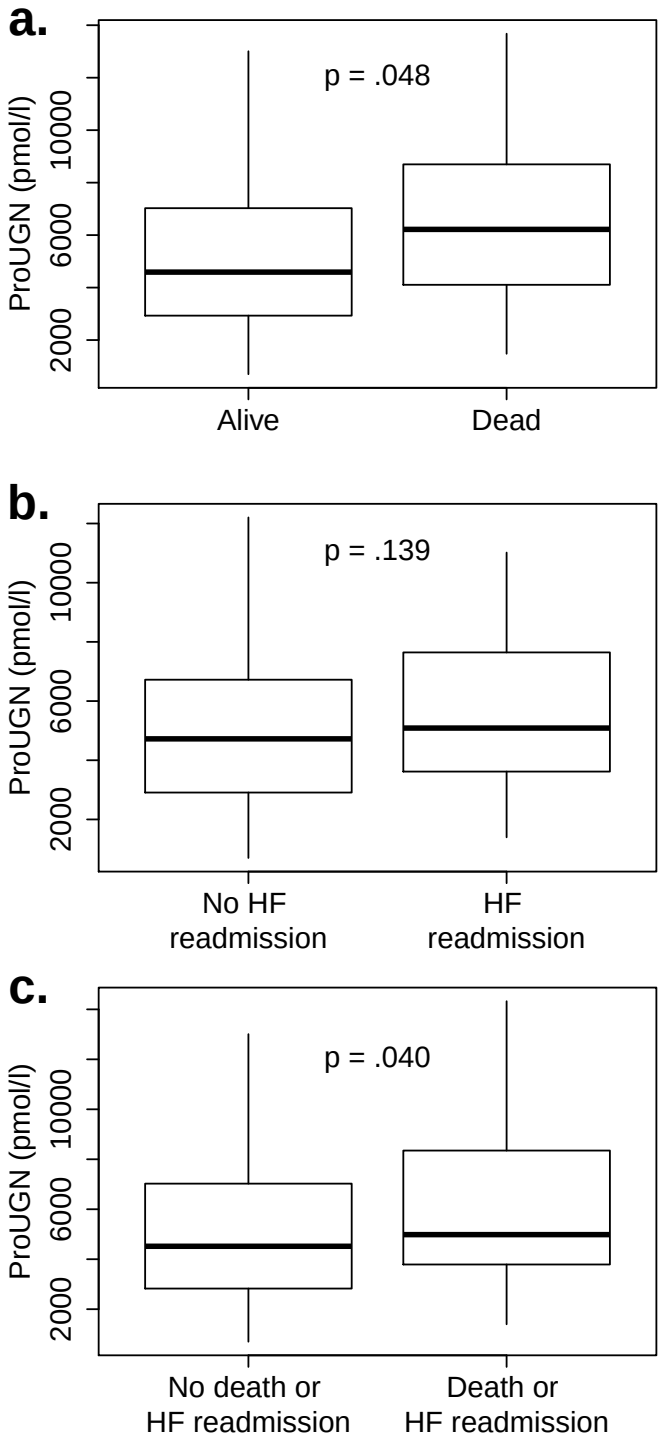

Figure 4.20 Pre-discharge ProUGN levels by outcome. ProUGN levels were significantly greater in those who died and in those with the combined endpoint. p values are shown for the t test.

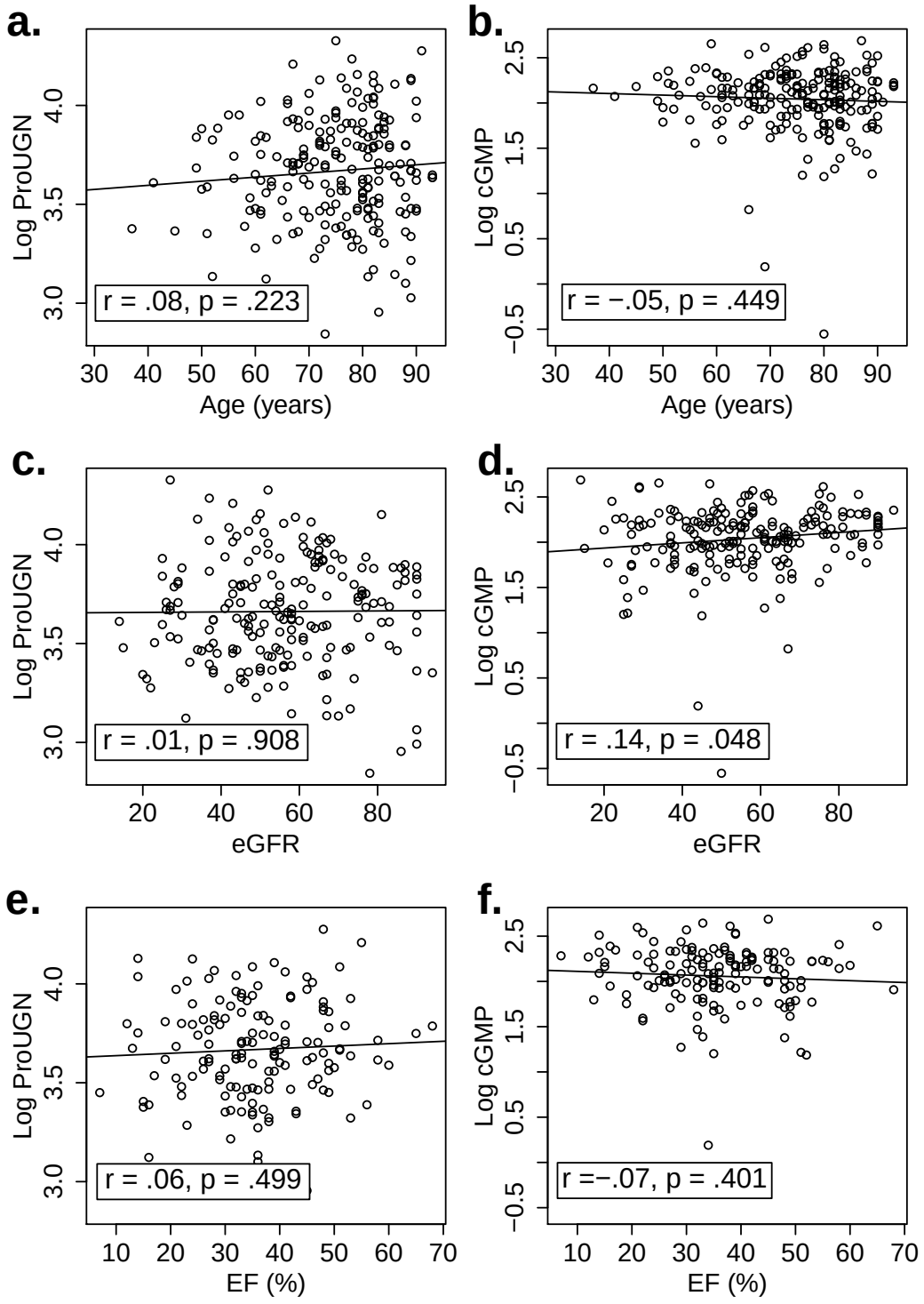

Figure 4.21 Scatter plots of pre-discharge ProUGN and cGMP. cGMP was significantly positively correlated with eGFR. Pearson's r coefficient is shown in the boxes.

cGMP

Univariate analysis of pre-discharge cGMP levels are shown in Table 4.7 and Figures 4.22, 4.23 and 4.24.

Pre-discharge cGMP was significantly less in those who had a HF readmission (96 [65 to 146] vs 146 [97 to 191] nmol·l^{-1}, p=.007). Pre-discharge cGMP significantly correlated with pre-discharge eGFR but not with age and EF (shown in Figure 4.21) or pre-discharge plasma sodium (r=.09, p=.194), LVIDS (r=.10, p=.214) or mean E/é (r=-.14, p=.221), plots not shown.

Table 4.7 Univariate analysis of pre-discharge cGMP in HF patients

	Pre-discharge cGMP[a] (nmol·l^{-1})	**p[b]**
Males *vs* females	120 (84 to 172) *vs* 152 (89 to 209)	.108
Past medical history *vs* none		
IHD	122 (64 to 183) *vs* 123 (85 to 174)	.260
Hypertension	120 (85 to 175) *vs* 146 (81 to 188)	.786
Diabetes	122 (85 to 180) *vs* 122 (85 to 181)	.762
AF	122 (66 to 159) *vs* 124 (87 to 189)	.034
Discharge medications *vs* none		
Aspirin	117 (85 to 187) *vs* 124 (85 to 174)	.535
Diuretic	122 (85 to 184) *vs* 110 (71 to 157)	.996
Betablocker	123 (89 to 186) *vs* 119 (72 to 167)	.922
ACEi or ARB	122 (87 to 181) *vs* 122 (60 to 179)	.954
CCB	132 (91 to 211) *vs* 122 (85 to 175)	.266
ARA	122 (85 to 182) *vs* 122 (85 to 178)	.677
Statin	121 (85 to 193) *vs* 126 (82 to 163)	.633
Nitrate	122 (85 to 204) *vs* 124 (85 to 171)	.933
Outcomes *vs* none		
Death	110 (54 to 159) *vs* 122 (85 to 183)	.165
HF readmission	96 (65 to 146) *vs* 146 (97 to 192)	.007
Death or HF readmission	101 (62 to 156) *vs* 133 (89 to 185)	.140

[a] Median (IQR)
[b] t test of log values

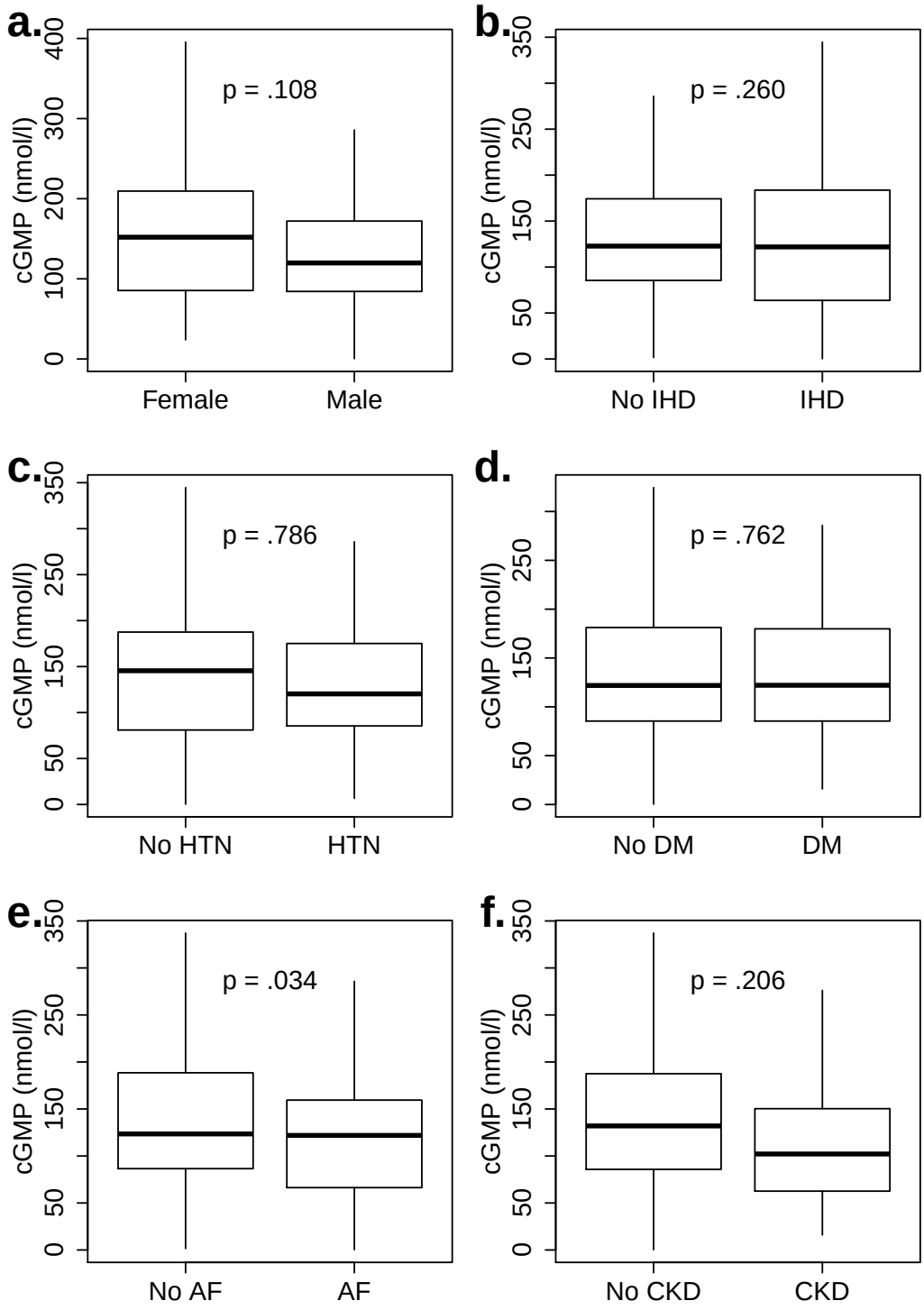

Figure 4.22 Pre-discharge cGMP levels by demographics and past medical history. cGMP levels were significantly lower in those with AF compared to those who did not. p values are shown for the t test.

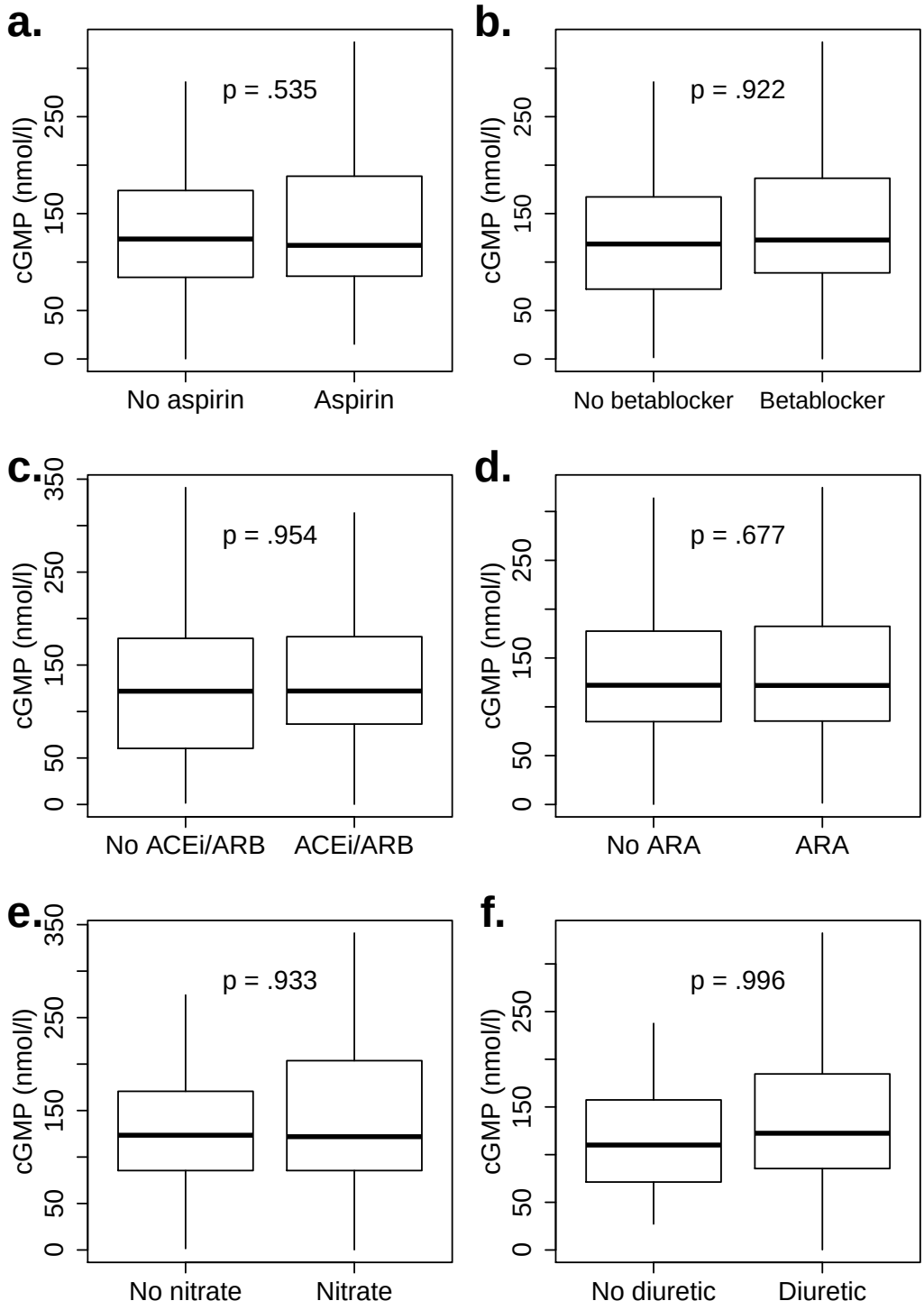

Figure 4.23 Pre-discharge cGMP levels by medications at discharge. cGMP levels were not significantly different in patients taking these medications compared to those not. p values are shown for the t test.

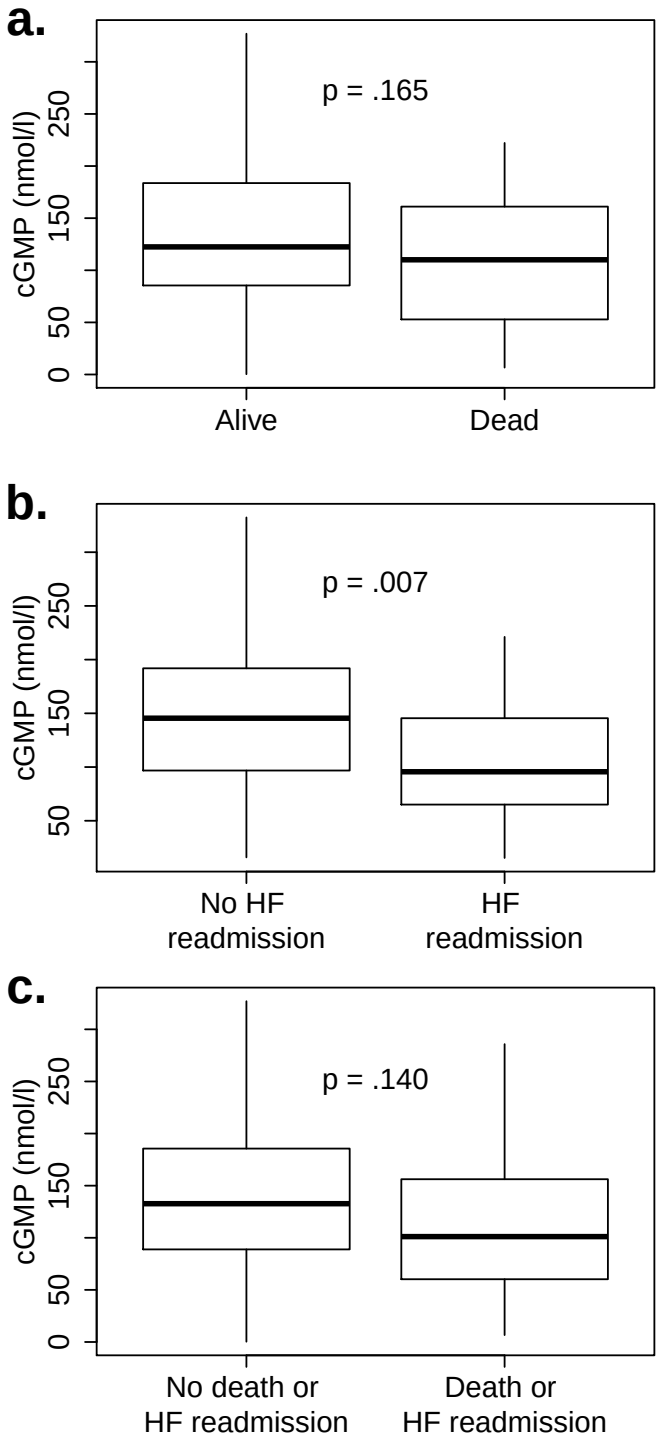

Figure 4.24 Pre-discharge cGMP levels by outcome. cGMP levels were significantly lower in those who were readmitted with AF compared to those who were not. p values are shown for the t test.

4.6.2 Logistic regression

Odds ratios with 95% C.I.s for variables predictive for the outcomes of all cause mortality, readmission with HF and all cause mortality or HF readmission using pre-discharge ProUGN, cGMP and NTproBNP are shown using forest plots in Figure 4.25.

Age (OR=1.06 [1.03 to 1.10], p<.001), ProUGN (OR=4.71 [1.04 to 23.5], p=.050, NTproBNP (OR=3.54 [1.52 to 9.26], p=.006) and a past history of IHD (OR=1.87 [1.08 to 3.27], p=.026) were associated with increased risk of all cause mortality while a higher eGFR (OR=0.97 [0.95 to 0.99], p=.001), taking a betablocker (OR=0.39 [0.22 to 0.67], p=.001), ACEi or ARB (OR=0.27 [0.15 to 0.47], p<.001) and diuretic (OR=0.25 [0.14 to 0.47], p<.001) were associated with a reduced risk of this outcome in univariate analysis (Figure 4.25a).

In multivariate analysis, higher NTproBNP levels (OR=3.46 [1.22 to 11.3], p=.029) remained independently associated with an increased risk of all cause mortality (Figure 4.25b).

Only a greater cGMP at discharge was associated with a reduced risk of HF readmission (OR=0.16 [0.04 to 0.63], p=.010) in univariate analysis (Figure 4.25c).

Greater age (OR=1.03 [1.01 to 1.05], p=.003) and higher ProUGN levels (OR=3.36 [1.07 to 11.2], p=.042), were significantly associated with increased risk of all cause mortality or HF readmission while a higher eGFR (OR=0.98 [0.97 to 0.99], p=.003), betablocker (OR=0.42 [0.26 to 0.68], p<.001), ACEi or ARB (OR=0.42 [0.26 to 0.68], p<.001) and diuretic use (OR=0.39 [0.22 to 0.69], p=.001) and were all associated with a reduced risk of this outcome in univariate analysis (Figure 4.25d).

In multivariate analysis higher ProUGN levels (OR=4.81 [1.28 to 19.7], p=.024) remained significantly associated with an increased risk of the combined endpoint while a higher eGFR (OR=0.97 [0.95 to 0.99], p=.011) was significantly associated with a reduced risk of this outcome (Figure 4.25e).

Pre-discharge ProUGN/cGMP ratio was significantly associated with increased risk of cause mortality (OR=2.47 [1.10 to 5.68], p=.028), readmission with HF (OR=4.65 [1.65 to 14.2], p=.005) and the combined endpoint

(OR=2.22 [1.15 to 4.49], p=.020) in univariate analysis. The pre-discharge NT-proBNP/cGMP ratio was significantly associated with increased risk of all cause mortality (OR=2.82 [1.48 to 5.70], p=.002) but not HF readmission alone (OR=1.50 [0.86 to 2.85], p=.183) or all cause mortality or HF readmission (OR=1.58 [1.02 to 2.55], p=.051) in univariate analysis.

4.6.3 Cox hazards regression

Hazard ratios (HR) with 95% C.I.s for variables predictive for the endpoints of time to all cause mortality, time to readmission with HF and time to all cause mortality or HF readmission are shown using forest plots in Figure 4.26.

Age (HR=1.06 [1.03 to 1.09], p<.001), NTproBNP (HR=3.38 [1.45 to 7.88], p=.005) and past history of IHD (HR=1.72 [1.05 to 2.82], p=.032) were all associated with increased risk of early mortality, while a higher eGFR (HR=0.97 [0.96 to 0.99], p<.001), betablocker (HR=0.40 [0.24 to 0.66], p<.001), ACEi or ARB (HR=0.29 [0.18 to 0.49], p<.001) and diuretic use (HR=0.28 [0.17 to 0.46], p<.001) were significantly associated with a reduced risk of early mortality in univariate analysis (Figure 4.26a).

In multivariate analysis only higher NTproBNP (HR=3.88 [1.33 to 11.3], p=.013) levels remained associated with a significantly increased risk of early mortality (Figure 4.26b).

Higher cGMP levels (HR=0.21 [0.07 to 0.64], p=.006) were associated with a reduced risk of early HF readmission in univariate analysis (Figure 4.26c).

Greater age (HR=1.02 [1.00 to 1.04], p=.022) and higher ProUGN levels (HR=2.63 [1.00 to 6.89], p=.049) were associated with significantly increased risk of early mortality or HF readmission while a higher eGFR (HR=0.98 [0.97 to 0.99], p=.002), betablocker (HR=0.46 [0.31 to 0.69], p<.001), ACEi or ARB (HR=0.46 [0.31 to 0.68], p<.001) and diuretic (HR=0.41 [0.27 to 0.63], p<.001) use were all associated with a reduced risk of the early combined endpoint (Figure 4.26d). In multivariate analysis higher ProUGN levels (HR=3.41 [1.17 to 9.93], p=.024) were associated with greater risk of the combined endpoint while a higher eGFR (HR=0.98 [0.96 to 0.99], p=.010) remained independently associated with a reduced risk of the early combined endpoint

(Figure 4.26e).

The pre-discharge ProUGN/cGMP ratio was significantly associated with early all cause mortality (HR=2.09 [1.12 to 3.89], p=.021) and the combined endpoint (HR=1.77 [1.11 to 2.81], p=.016) as well as early readmission with HF alone (HR=3.87 [1.59 to 9.41], p=.003) in univariate analysis. The pre-discharge NTproBNP/cGMP ratio was significantly associated with early all cause mortality (HR=2.44 [1.41 to 4.20], p=.001), early all cause mortality or HF readmission (HR=1.48 [1.01 to 2.18], p=.045), but not HF readmission alone (HR=1.50 [0.85 to 2.62], p=.159) in univariate analysis.

4.6.4 Kaplan-Meier analysis

Kaplan-Meier plots stratified by median pre-discharge NTproBNP, ProUGN and cGMP level for outcomes of all cause mortality, HF readmission and all cause mortality or HF readmission are shown in Figures 4.27, 4.28 and 4.29.

Lower pre-discharge levels of cGMP were significantly associated with an increased risk of HF readmission while showing a trend to increased risk of all cause mortality or HF readmission. A lower pre-discharge NTproBNP/cGMP ratio was significantly associated with event free survival for all outcomes while a lower pre-discharge ProUGN/cGMP ratio was associated with reduced risk of HF readmission (Figures 4.30 and 4.31).

4.6.5 ROC curve analysis

Classification by outcome for pre-discharge ProUGN, NTproBNP, cGMP as well as ProUGN/cGMP and NTproBNP/cGMP ratios is shown using ROC curves in Figure 4.32. NTproBNP (AUC=.673 [.561 to .786]) did not have a significantly greater classification accuracy compared to ProUGN (AUC=.622 [.516 to .727], p=.497) and cGMP (inverse AUC=.575 [.449 to .702], p=.241) for outcome all cause mortality (Figure 4.32a). NTproBNP (AUC=.542 [.431 to .654]) did not significantly classify for outcome HF readmission any better than ProUGN (AUC=.594 [.492 to .695], p=.537) or cGMP (inverse AUC=.616 [.509 to .722], p=.270) (Figure 4.32b). For the combined endpoint of all cause mortality or

HF readmission, NTproBNP (AUC=.595 [.505 to .684]) was not significantly better at classification compared to cGMP (inverse AUC=.589 [.499 to .679], p=.880) or ProUGN (AUC=.598 [.514 to .682], p=.993) (Figure 4.32c).

A greater pre-discharge NTproBNP/cGMP ratio (AUC=.692 [.589 to .796]) did not have significantly greater classification accuracy than ProUGN/cGMP ratio (AUC=.629 [.505 to .752], p=.287) for outcome all cause mortality, HF readmission (AUC=.586 [.471 to .701] vs AUC=.633 [.528 to .739], p=.430) or the combined endpoint (AUC=.628 [.540 to .715] vs AUC=.620 [.531 to .708], p=.836).

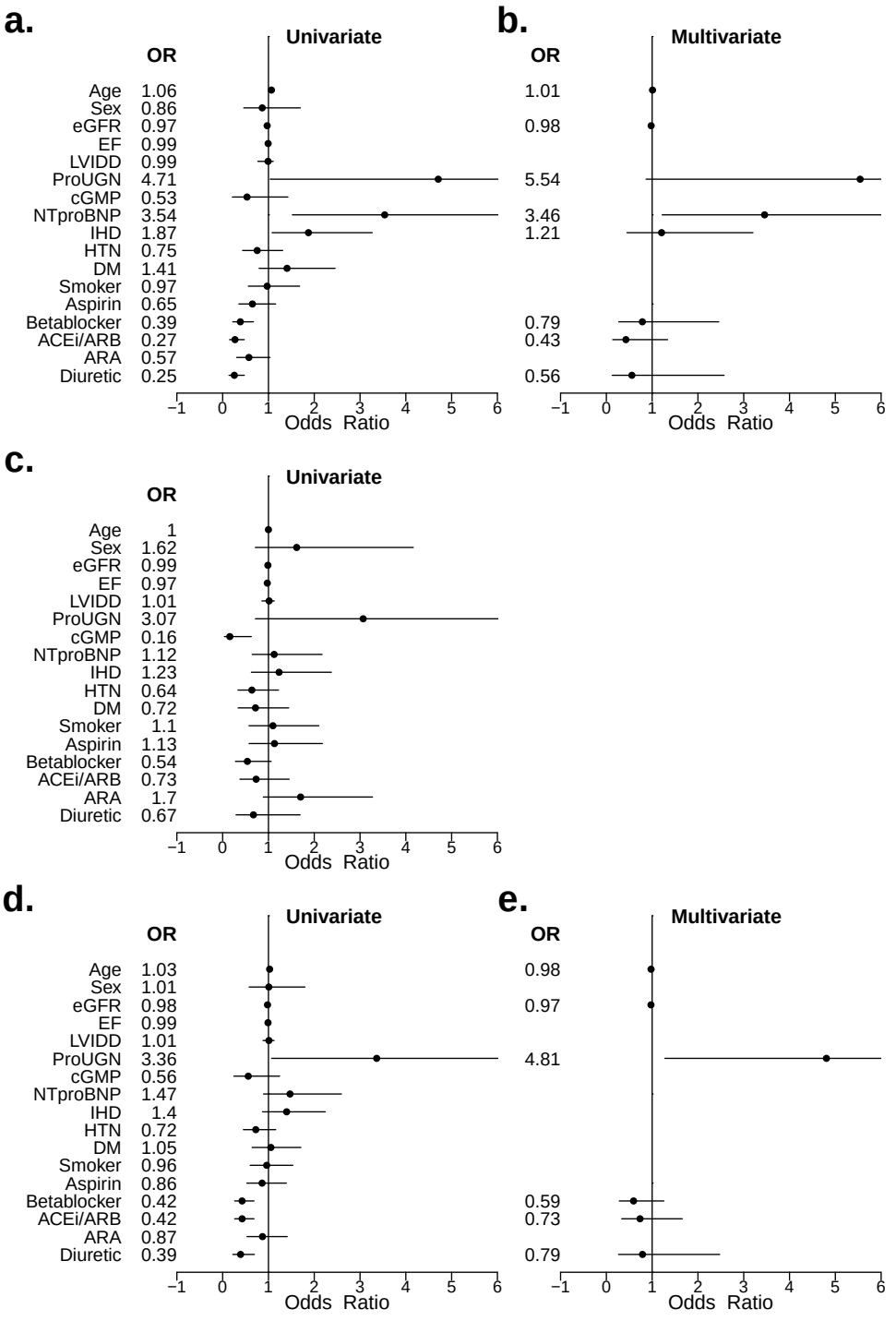

Figure 4.25 Forest plots showing odds ratios with 95% C.I.s for logistic regression analysis of pre-discharge ProUGN, NTproBNP and cGMP for outcomes: All cause mortality (**a** & **b**), HF readmission (**c**) and all cause mortality or HF readmission (**d** & **e**). Pre-discharge eGFR and medications on discharge are used.

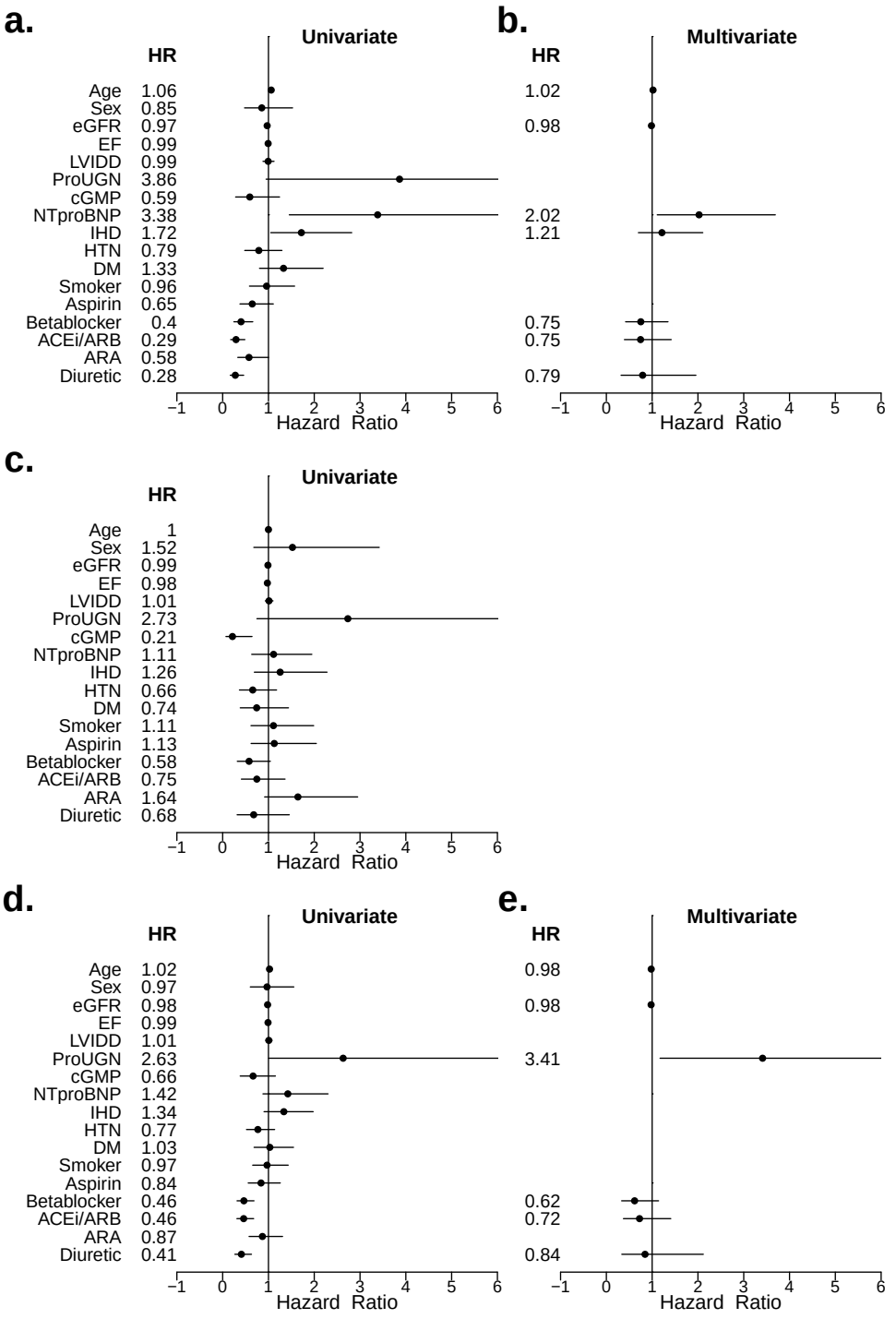

Figure 4.26 Forest plots showing Hazard ratios with 95% C.I.s for Cox hazards regression analysis of pre-discharge ProUGN, NTproBNP and cGMP for outcomes: All cause mortality (**a** & **b**), HF readmission (**c**) and all cause mortality or HF readmission (**d** & **e**). Pre-discharge eGFR and medications on discharge are used.

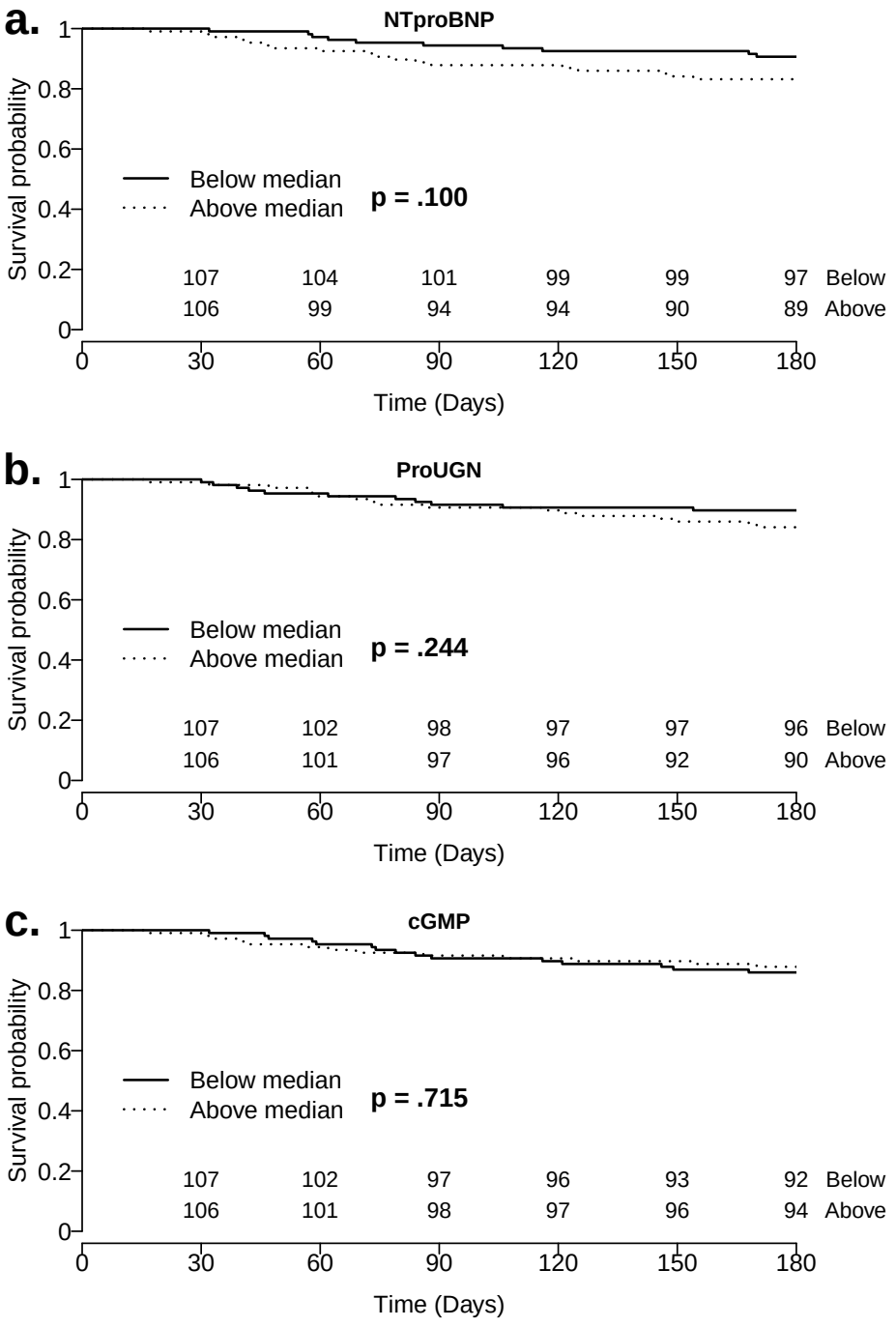

Figure 4.27 Kaplan-Meier plots for outcome all cause mortality stratified by pre-discharge **a.** NTproBNP **b.** ProUGN **c.** cGMP. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

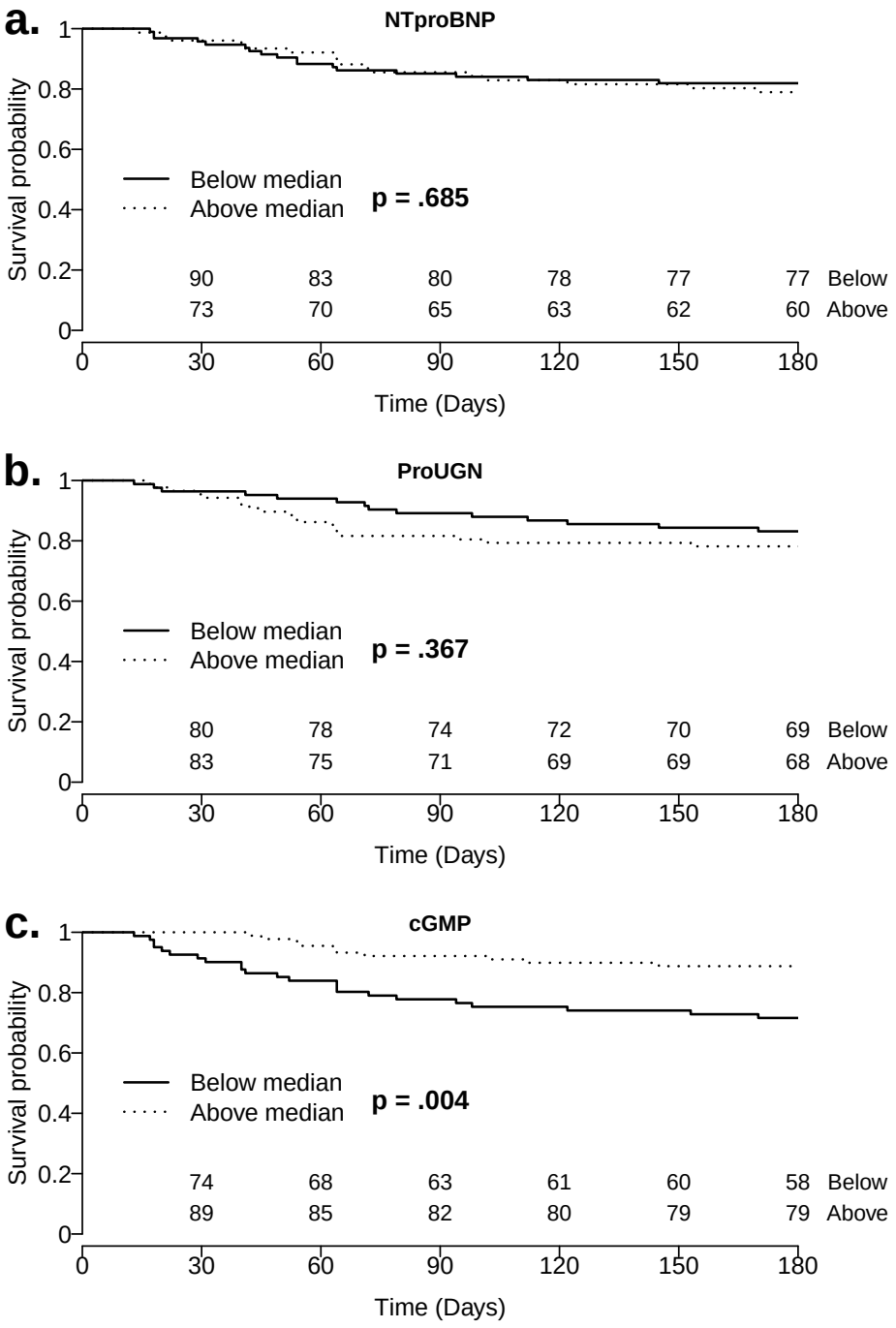

Figure 4.28 Kaplan-Meier plots for outcome of HF readmission stratified by pre-discharge **a.** NTproBNP **b.** ProUGN **c.** cGMP. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

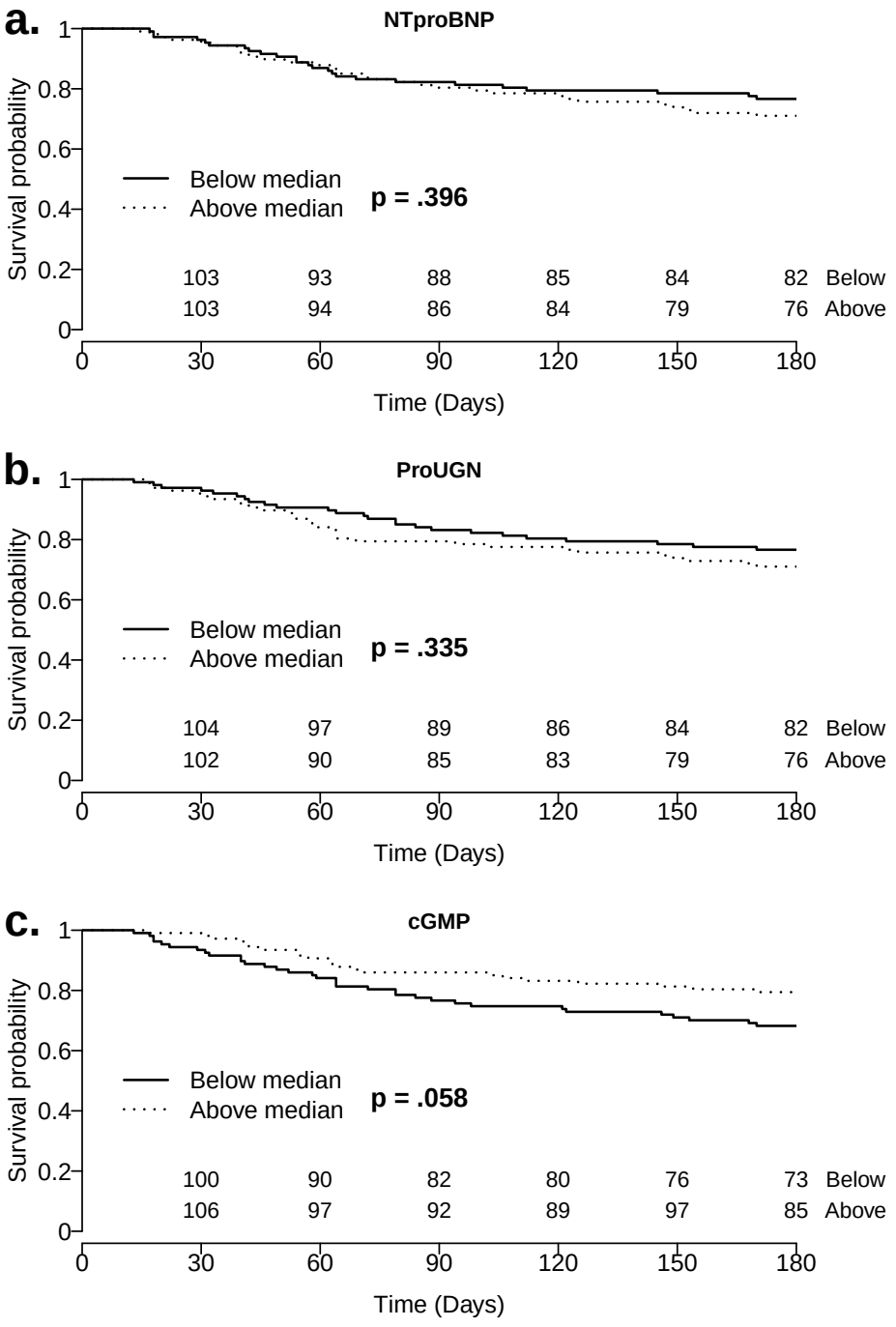

Figure 4.29 Kaplan-Meier plots for outcome all cause mortality or HF readmission stratified by pre-discharge **a.** NTproBNP **b.** ProUGN **c.** cGMP. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

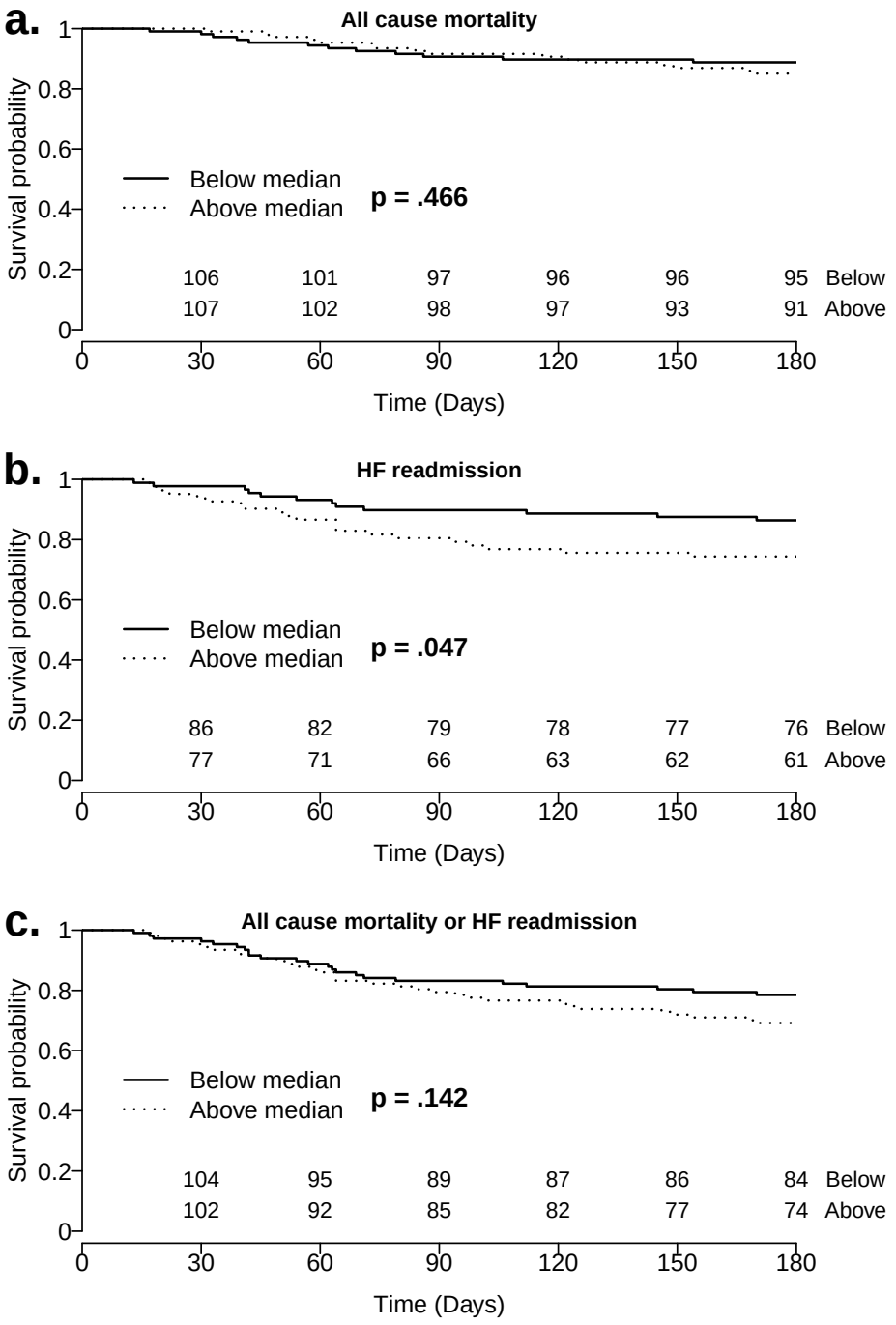

Figure 4.30 Kaplan-Meier plots stratified by median pre-discharge ProUGN/cGMP ratio for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

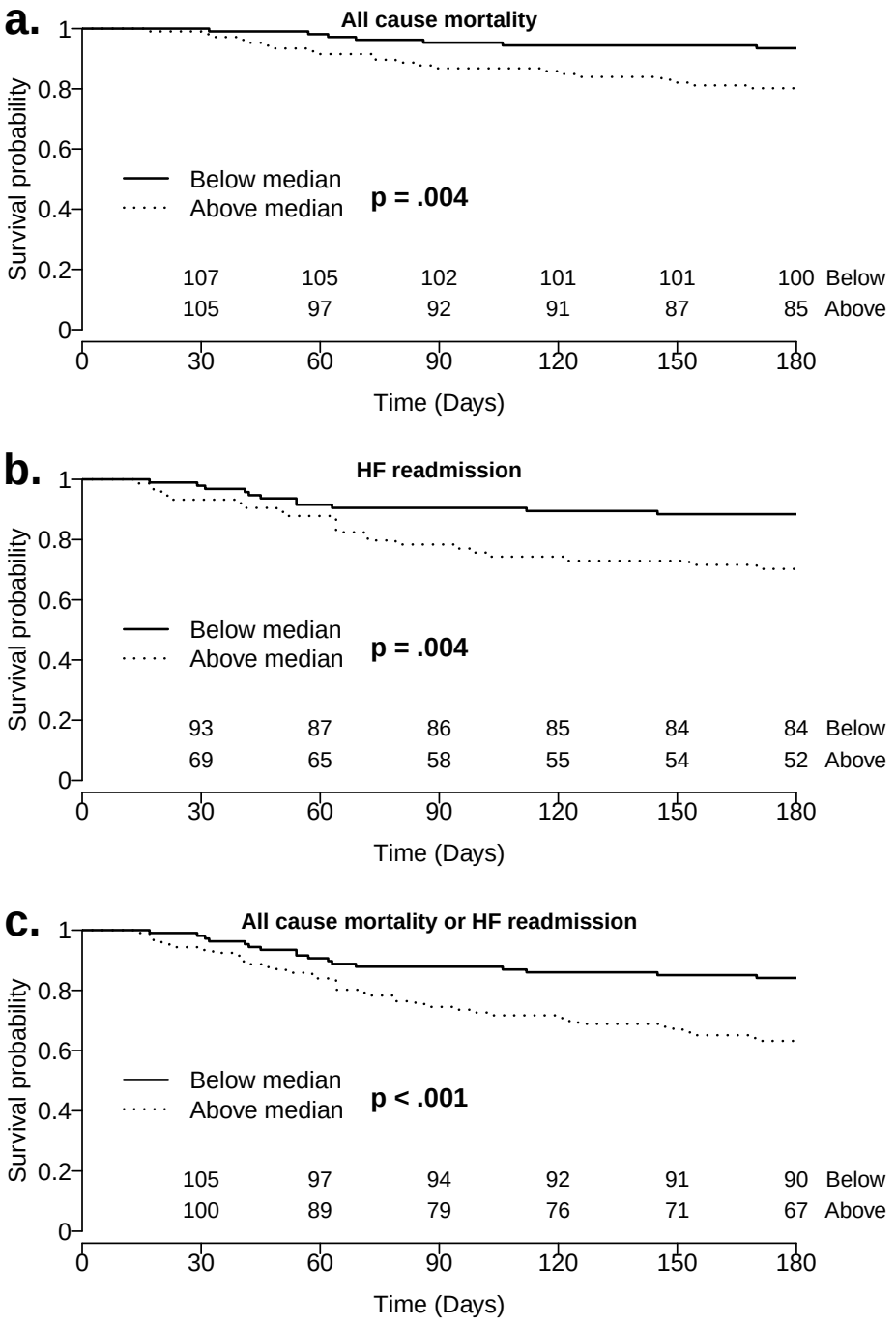

Figure 4.31 Kaplan-Meier plots stratified by median pre-discharge NT-proBNP/cGMP ratio for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

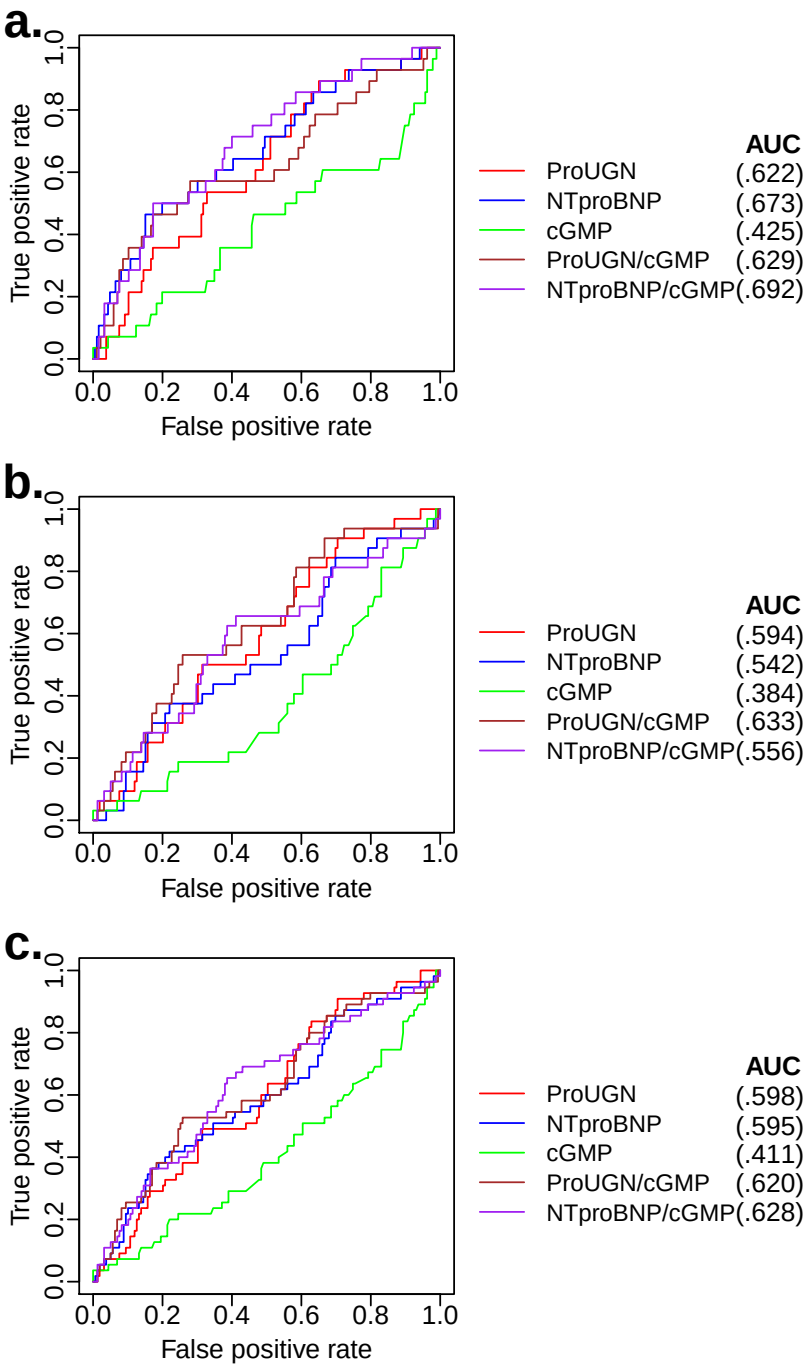

Figure 4.32 ROC curves for pre-discharge biomarkers for endpoints **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. AUC values are shown in brackets.

4.7 Pre-discharge *vs* admission comparison

4.7.1 Linear analysis

Absolute levels

Changes in measured variables at admission compared to pre-discharge are shown using box plots in Figure 4.33. Pre-discharge ProUGN levels were significantly greater than at admission (4740 [2983 to 7390] vs 1297 [904 to 1763] pmol·l^{-1}, p<.001) as were cGMP levels (122 [85 to 181] vs 109 [66 to 148] nmol·l^{-1}, p=.045), while NTproBNP was significantly lower (1848 [736 to 3869] vs 2942 [1439 to 5709] pmol·l^{-1}, p<.001). Weight significantly decreased during hospital stay (87.8 [73 to 105.2] kg at admission vs 82.0 [71.8 to 103.7] kg at pre-discharge, p<.001) but there were no significant change in eGFR and plasma sodium.

Individual changes in measured variables colour coded by outcome are shown using line plots in Figures 4.34, 4.35 and 4.36 but show no clearly visible trends.

There were no significant relationship between plasma ProUGN and NT-proBNP levels to cGMP at admission and pre-discharge (Figure 4.39a-d). There were no significant differences in the relationship between ProUGN and cGMP stratified by outcome of all cause mortality of HF readmission (Figure 4.40a-d).

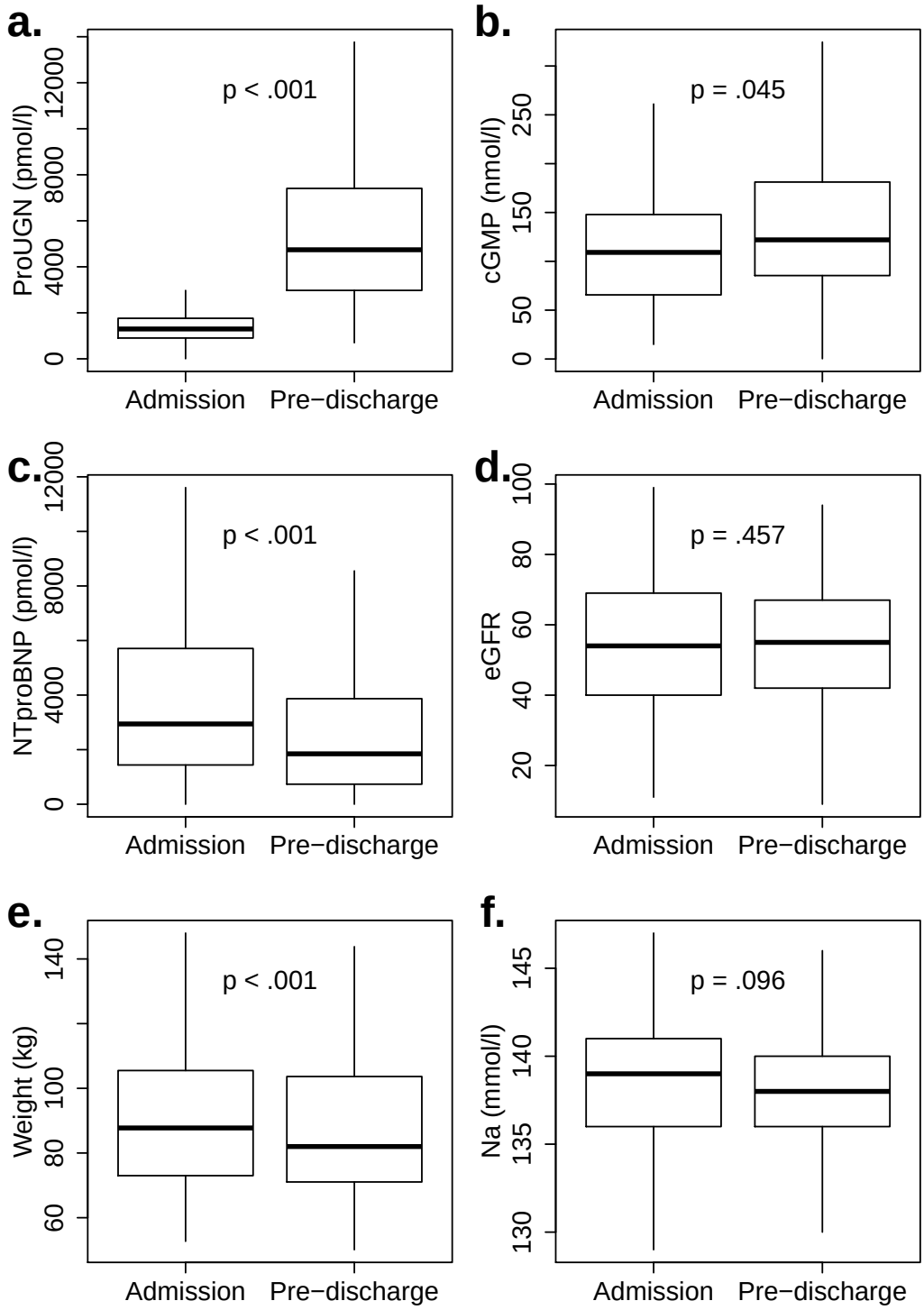

Figure 4.33 Box plots comparing variables at admission and pre-discharge. ProUGN and cGMP were both significantly greater pre-discharge compared to at admission while NTproBNP and patient weight were significantly lower. p values are shown for the paired t test.

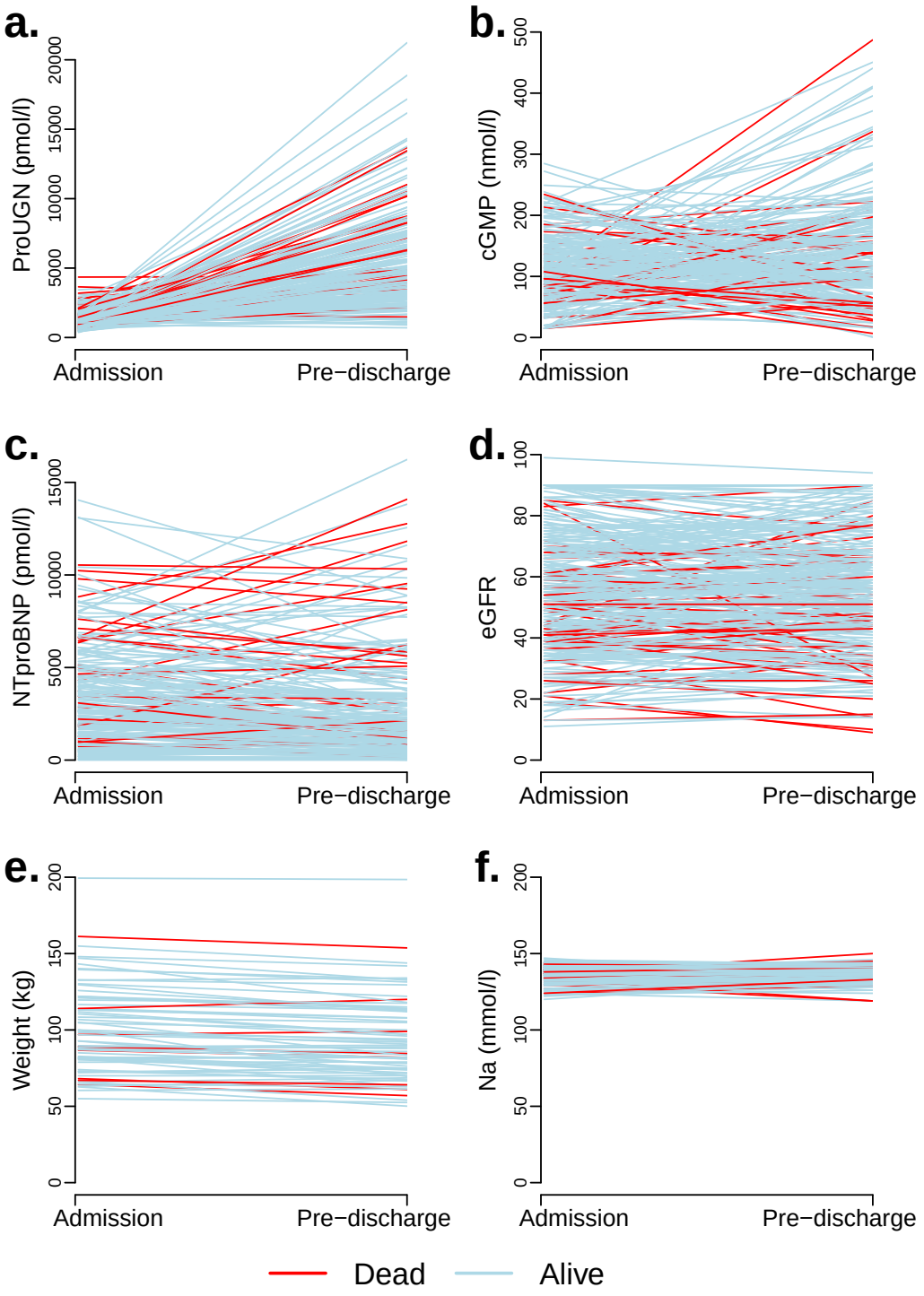

Figure 4.34 Line plots showing individual changes in variables between admission and pre-discharge, colour coded for the outcome of all cause mortality. Each line represents a single person.

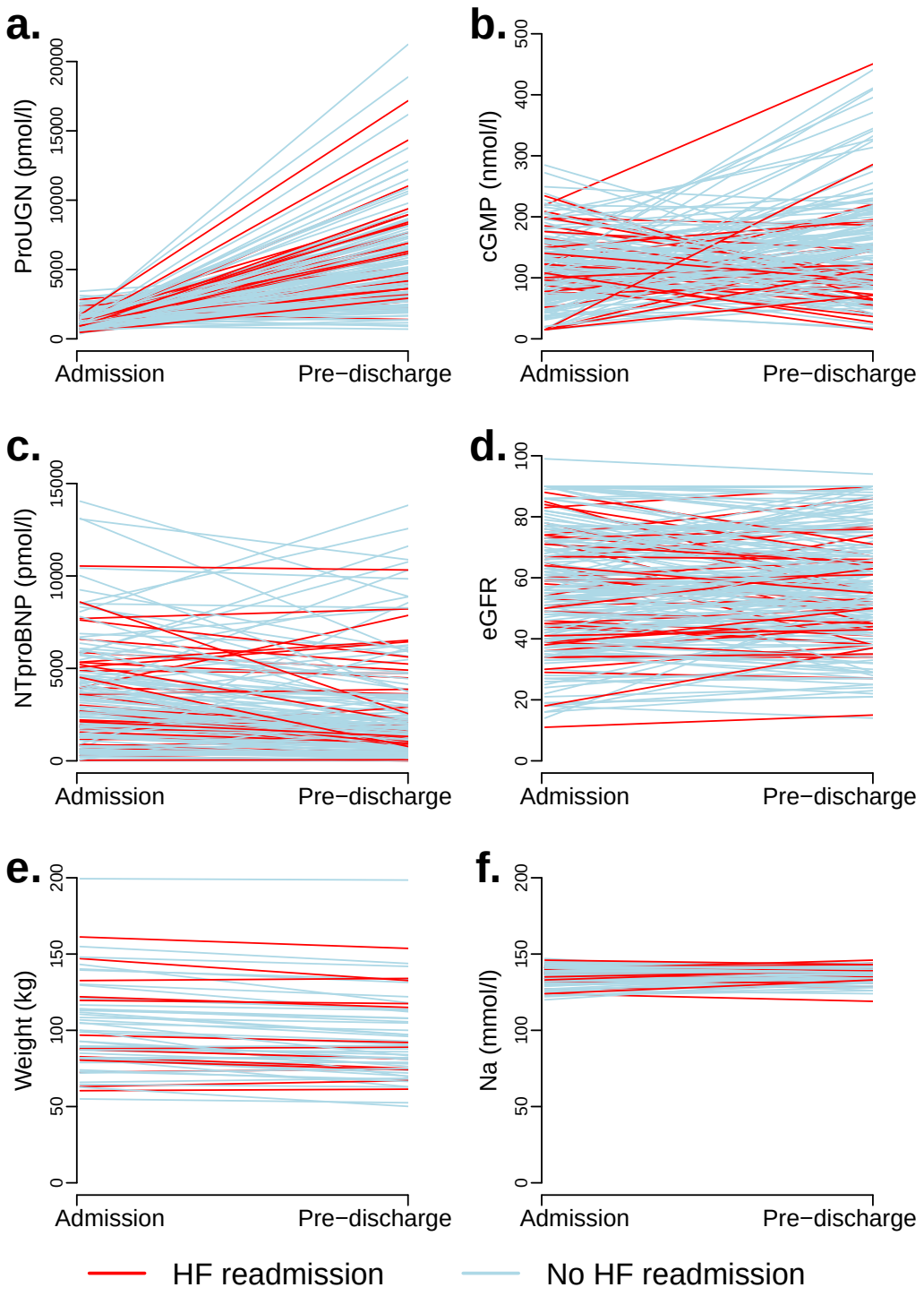

Figure 4.35 Line plots showing individual changes in variables between admission and pre-discharge, colour coded for the outcome of readmission with HF. Each line represents a single person.

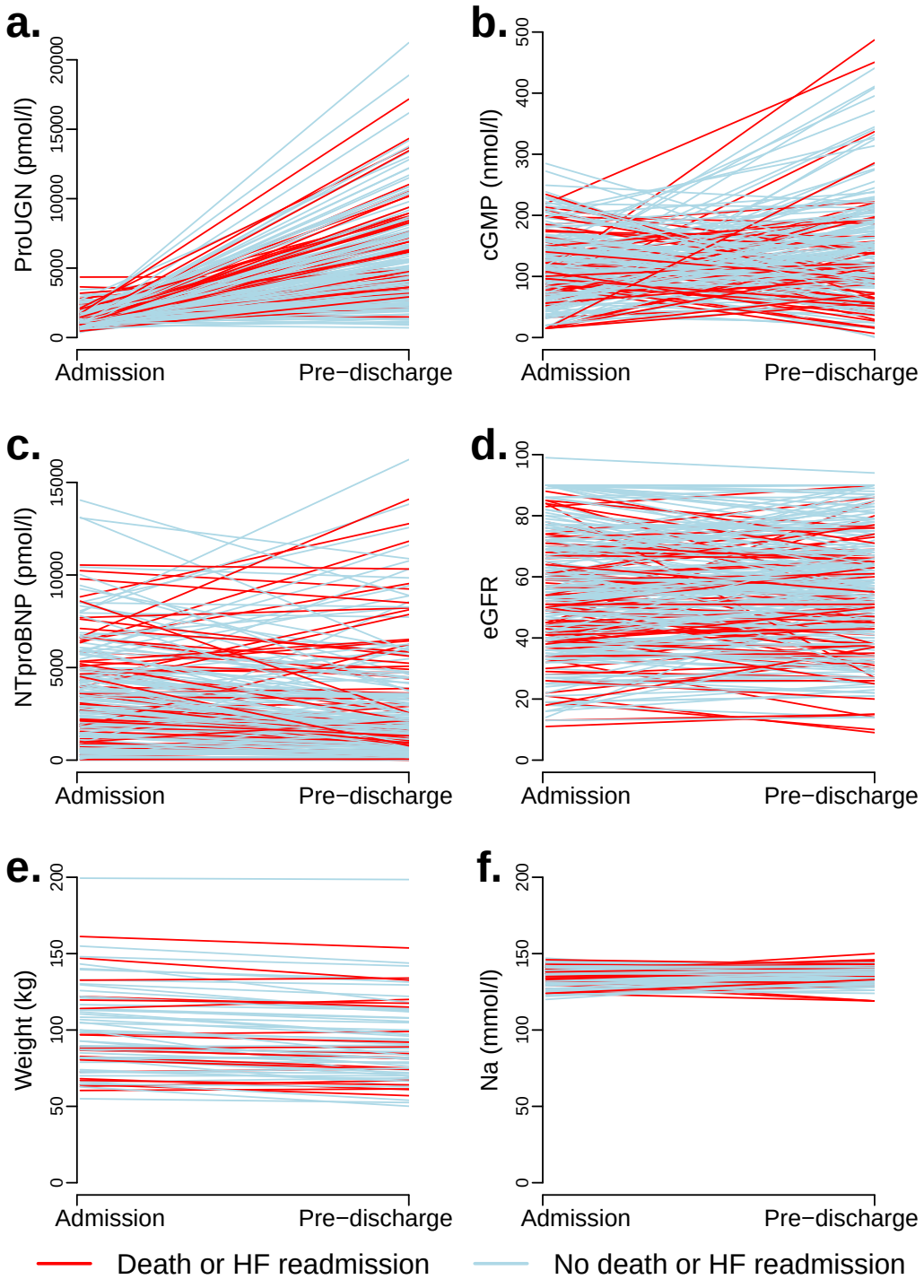

Figure 4.36 Line plots showing individual changes in variables between admission and pre-discharge, colour coded for the outcome of all cause mortality or HF readmission. Each line represents a single person.

Pre-discharge/admission ratio

Univariate analysis of pre-discharge/admission ratios of ProUGN and cGMP is shown in Tables 4.8 and 4.9 with outcomes illustrated in Figures 4.37 and 4.38. Pre-discharge cGMP levels were significantly less compared to at admission in those who had all the outcomes. NTproBNP levels were significantly greater pre-discharge compared to at admission in those who died, while the eGFR pre-discharge/admission ratio was not significantly different for any outcome.

There were no significant relationships between pre-discharge/admission ProUGN and NTproBNP ratios to pre-discharge/admission cGMP ratio (Figure 4.39e-f). There were no significant differences in the relationship between pre-discharge/admission ProUGN ratio and pre-discharge/admission cGMP ratio stratified by outcome of all cause mortality of HF readmission (Figure 4.40e-f).

Table 4.8 Univariate analysis of ratio of pre-discharge to admission ProUGN in HF patients

	ProUGN ratio[a]	**p**[b]
Males *vs* females	4.1 (2.3 to 6.8) *vs* 2.5 (1.5 to 4.6)	.002
Past medical history *vs* none		
IHD	4.3 (2.4 to 6.6) *vs* 3.4 (1.9 to 6.4)	.308
Hypertension	3.6 (1.7 to 6.6) *vs* 3.9 (2.4 to 6.4)	.203
Diabetes	3.5 (2.1 to 5.6) *vs* 4.0 (1.9 to 6.8)	.751
AF	3.6 (1.8 to 6.0) *vs* 3.8 (2.1 to 7.2)	.291
Admission medications *vs* none		
Aspirin	4.0 (1.9 to 6.6) *vs* 3.6 (1.9 to 6.4)	.674
Diuretic	3.9 (2.1 to 6.6) *vs* 3.7 (1.8 to 5.9)	.296
Betablocker	3.5 (1.8 to 6.3) *vs* 4.1 (2.1 to 6.6)	.349
ACEi or ARB	3.7 (1.8 to 6.4) *vs* 3.8 (2.1 to 6.5)	.638
CCB	3.3 (2.0 to 4.6) *vs* 4.0 (1.9 to 6.9)	.154
ARA	4.5 (2.1 to 6.8) *vs* 3.7 (1.9 to 6.4)	.437
Statin	3.7 (1.9 to 5.5) *vs* 3.8 (2.0 to 7.1)	.244
Outcomes *vs* none		
Death	4.3 (2.3 to 6.5) *vs* 3.7 (1.9 to 6.5)	.739
HF readmission	5.2 (2.5 to 6.9) *vs* 3.7 (1.9 to 6.2)	.234
Death or HF readmission	4.4 (2.3 to 6.7) *vs* 3.6 (1.9 to 6.0)	.307

[a] Median (IQR)
[b] t test of log values

Table 4.9 Univariate analysis of ratio of pre-discharge to admission cGMP in HF patients

	cGMP ratio[a]	**p**[b]
Males *vs* females	1.2 (0.6 to 2.3) *vs* 1.4 (0.8 to 2.0)	.466
Past medical history *vs* none		
IHD	1.2 (0.6 to 2.8) *vs* 1.2 (0.6 to 2.1)	.646
Hypertension	1.1 (0.5 to 1.9) *vs* 1.4 (0.7 to 3.0)	.262
Diabetes	1.2 (0.6 to 1.9) *vs* 1.2 (0.7 to 2.3)	.840
AF	1.2 (0.6 to 2.4) *vs* 1.2 (0.7 to 1.9)	.636
Admission medications *vs* none		
Aspirin	1.1 (0.6 to 1.8) *vs* 1.3 (0.7 to 2.4)	.450
Diuretic	1.1 (0.5 to 1.9) *vs* 1.4 (0.7 to 2.7)	.041
Betablocker	1.2 (0.6 to 2.2) *vs* 1.2 (0.6 to 2.5)	.958
ACEi or ARB	1.2 (0.6 to 2.3) *vs* 1.3 (0.7 to 2.0)	.584
CCB	1.3 (0.5 to 1.8) *vs* 1.2 (0.7 to 2.3)	.320
ARA	1.1 (0.4 to 1.8) *vs* 1.2 (0.7 to 2.4)	.079
Statin	1.2 (0.6 to 2.0) *vs* 1.2 (0.6 to 2.4)	.659
Outcomes *vs* none		
Death	1.2 (0.5 to 1.7) *vs* 1.2 (0.7 to 2.3)	.401
HF readmission	0.7 (0.5 to 1.6) *vs* 1.4 (0.8 to 2.5)	.056
Death or HF readmission	1.1 (0.5 to 1.7) *vs* 1.3 (0.7 to 2.4)	.450

[a] Median (IQR)
[b] t test of log values

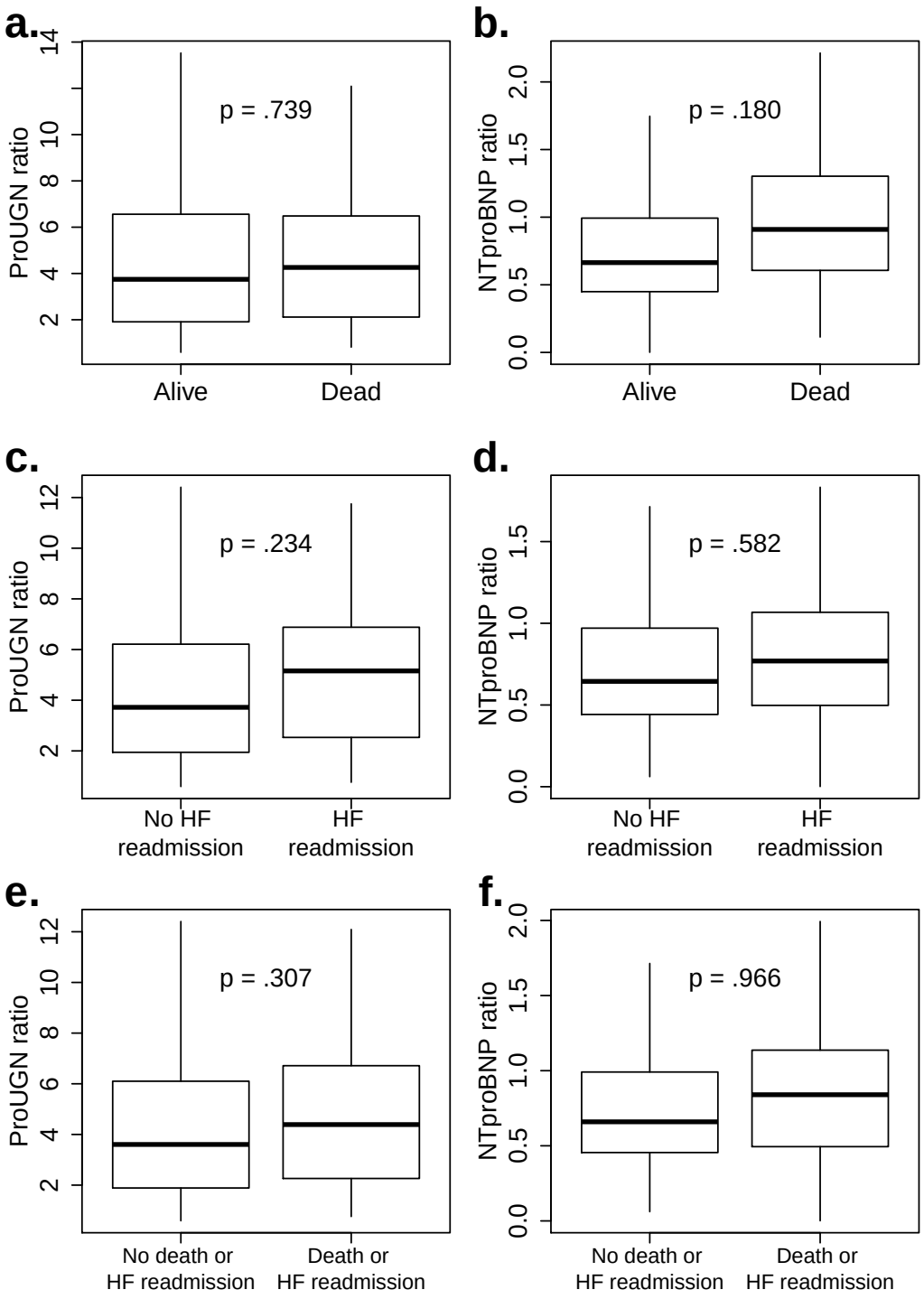

Figure 4.37 Box plots comparing outcomes for ProUGN and NTproBNP pre-discharge/admission ratios. There were no significant differences between pre-discharge/admission ratio for either ProUGN or NTproBNP for any outcome. p values are shown for the t test.

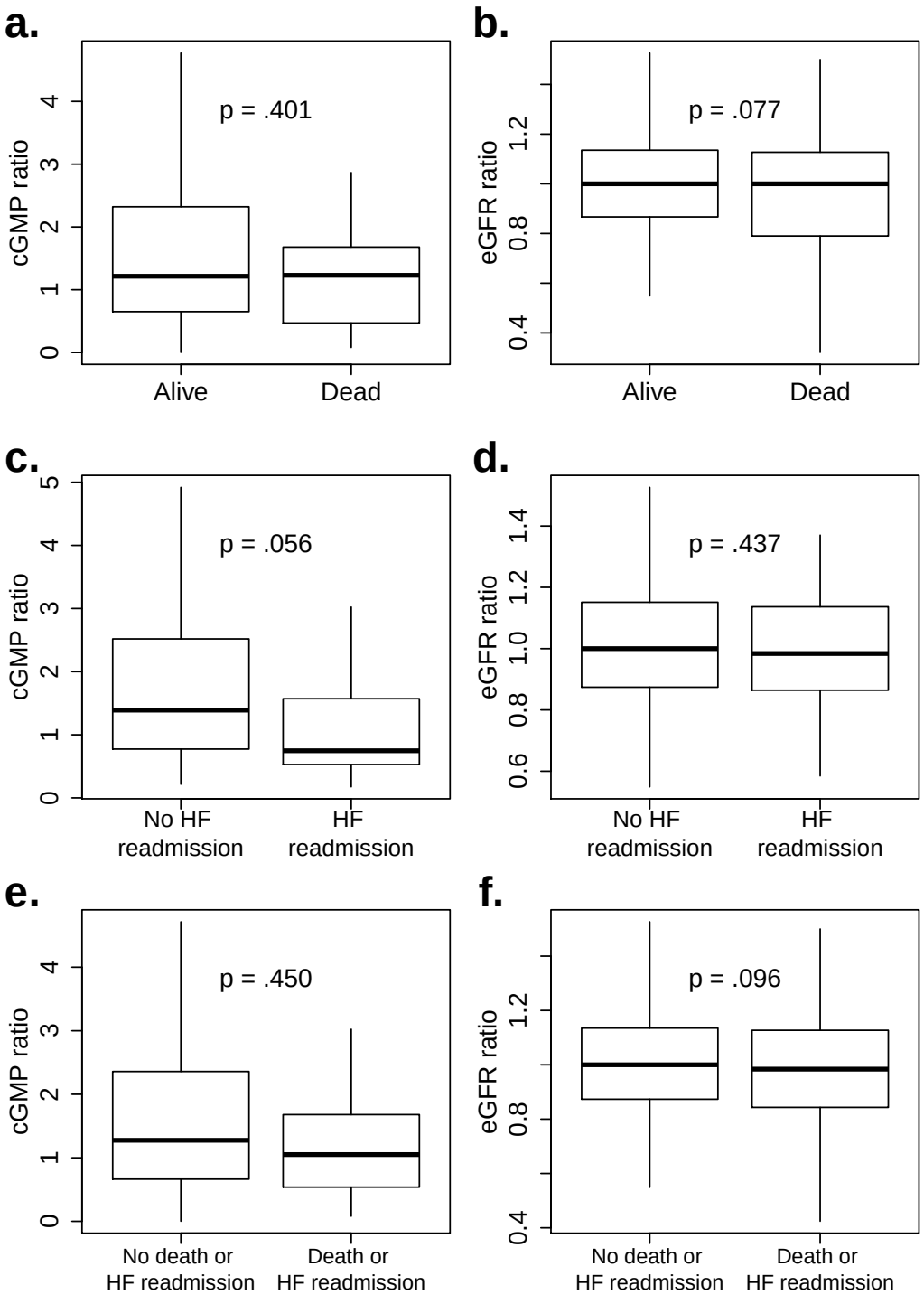

Figure 4.38 Box plots comparing outcomes for cGMP and eGFR pre-discharge/admission ratios.There were no significant differences between pre-discharge/admission ratio for either cGMP or eGFR for any outcome. p values are shown for the t test.

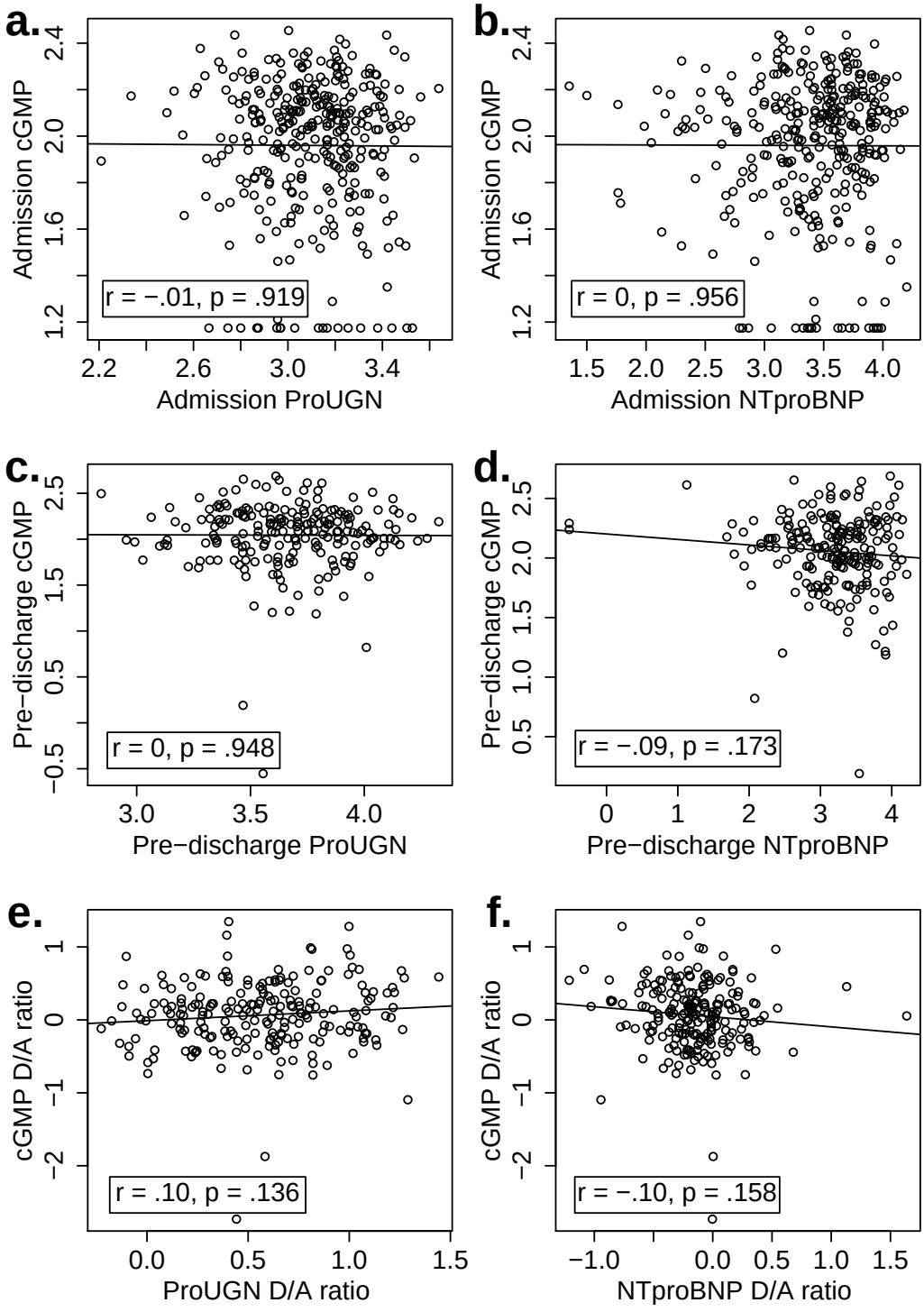

Figure 4.39 Scatter plots of admission and pre-discharge ProUGN,NTproBNP and cGMP. Pearson's r coefficient is shown in the text boxes.

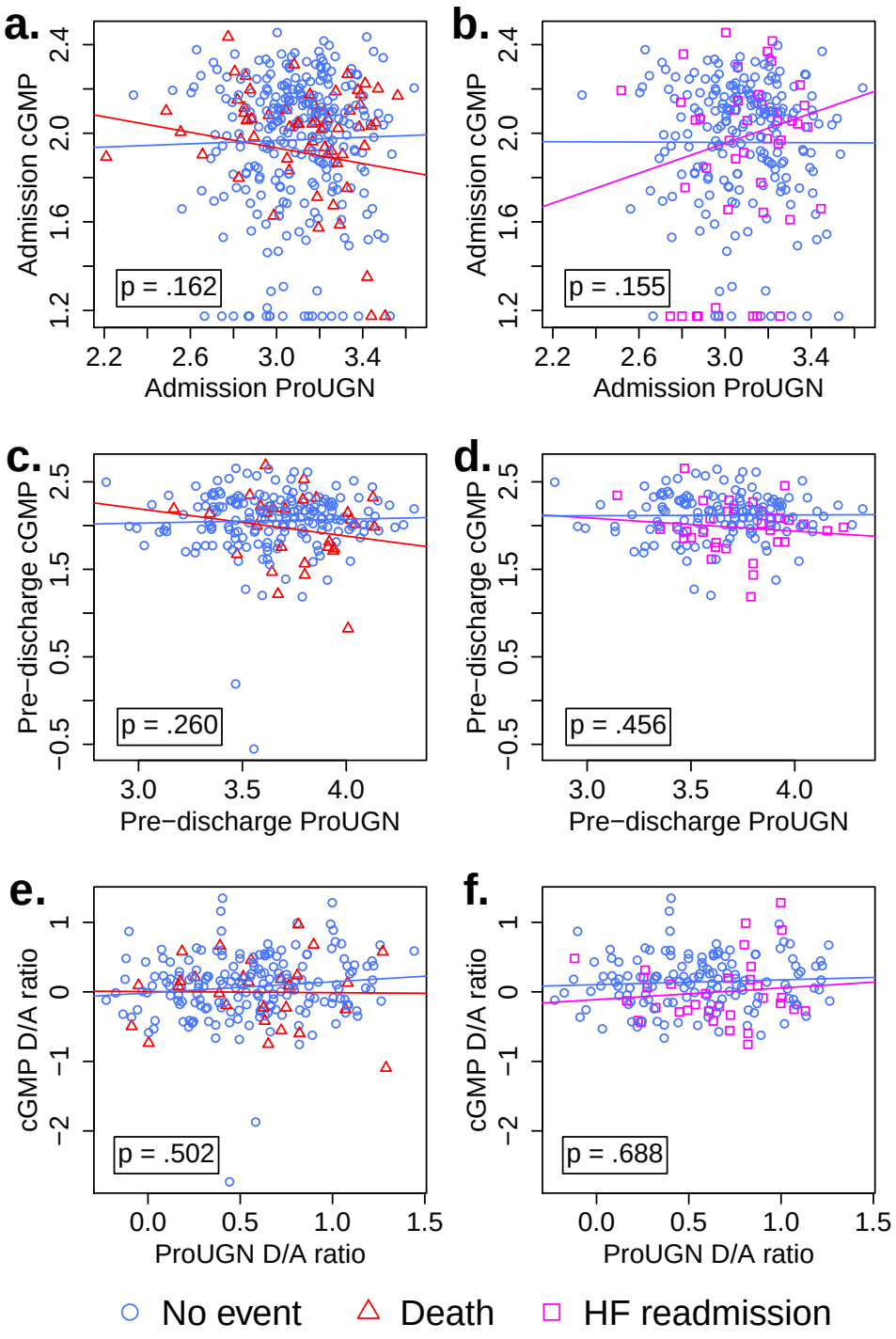

Figure 4.40 Comparison of admission cGMP and ProUGN linear regression by outcome. Log values were used. p values for the difference between regression slopes are shown in the text boxes.

Peptide/cGMP ratio

The differences in relative levels of ProUGN and NTproBNP compared to cGMP at admission and discharge for different outcomes are shown in Figure 4.41 and 4.42. The pre-discharge ProUGN/cGMP ratio was significantly greater in those that had all outcomes, but no significant difference was observed at admission (Figure 4.41). The NTproBNP/cGMP ratio at admission and pre-discharge was significantly greater in those with outcomes all cause mortality and HF readmission or all cause mortality at both admission and pre-discharge (Figure 4.42).

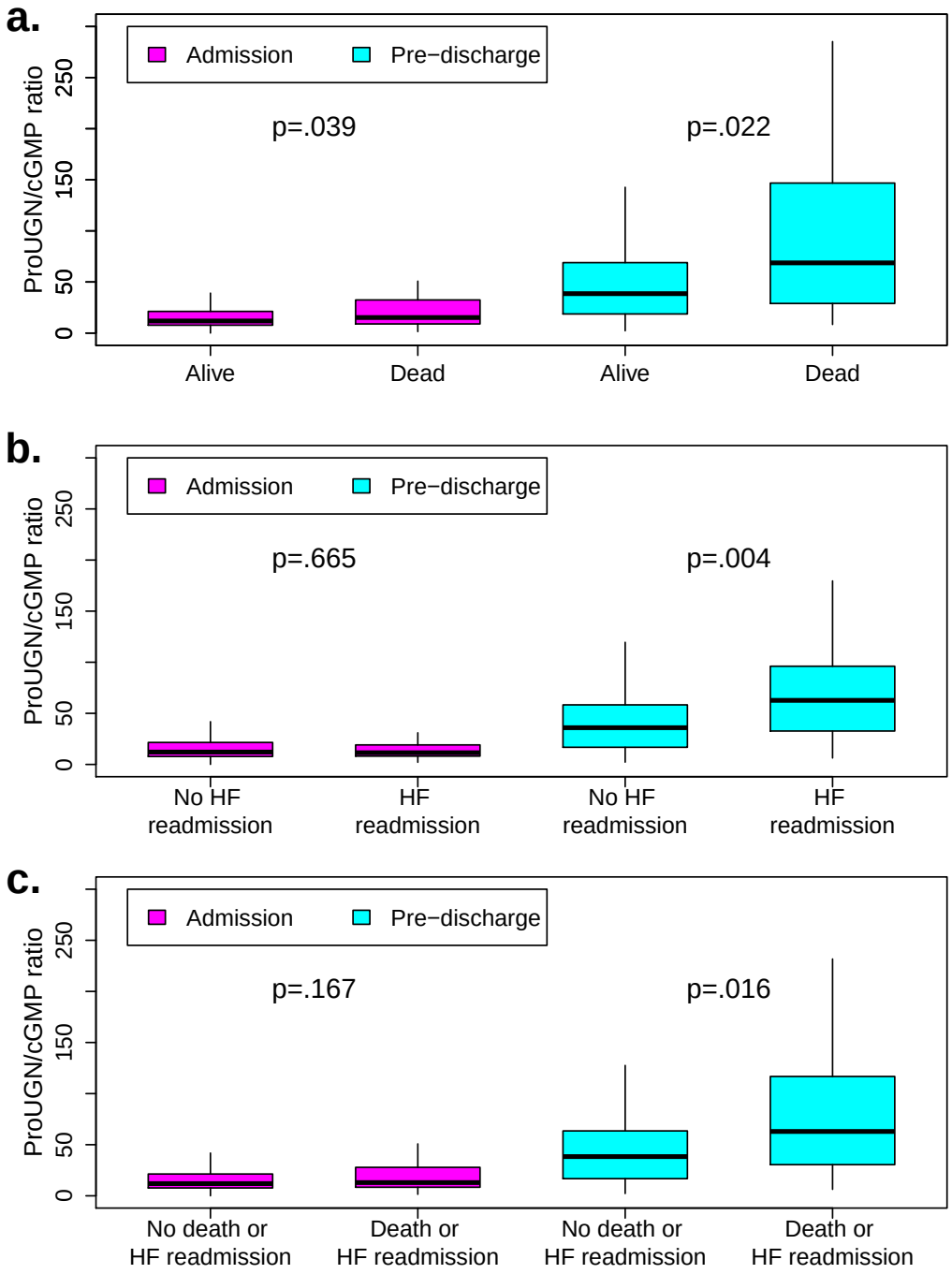

Figure 4.41 Comparison of ProUGN/cGMP ratio at admission and pre-discharge for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values are shown for the t test.

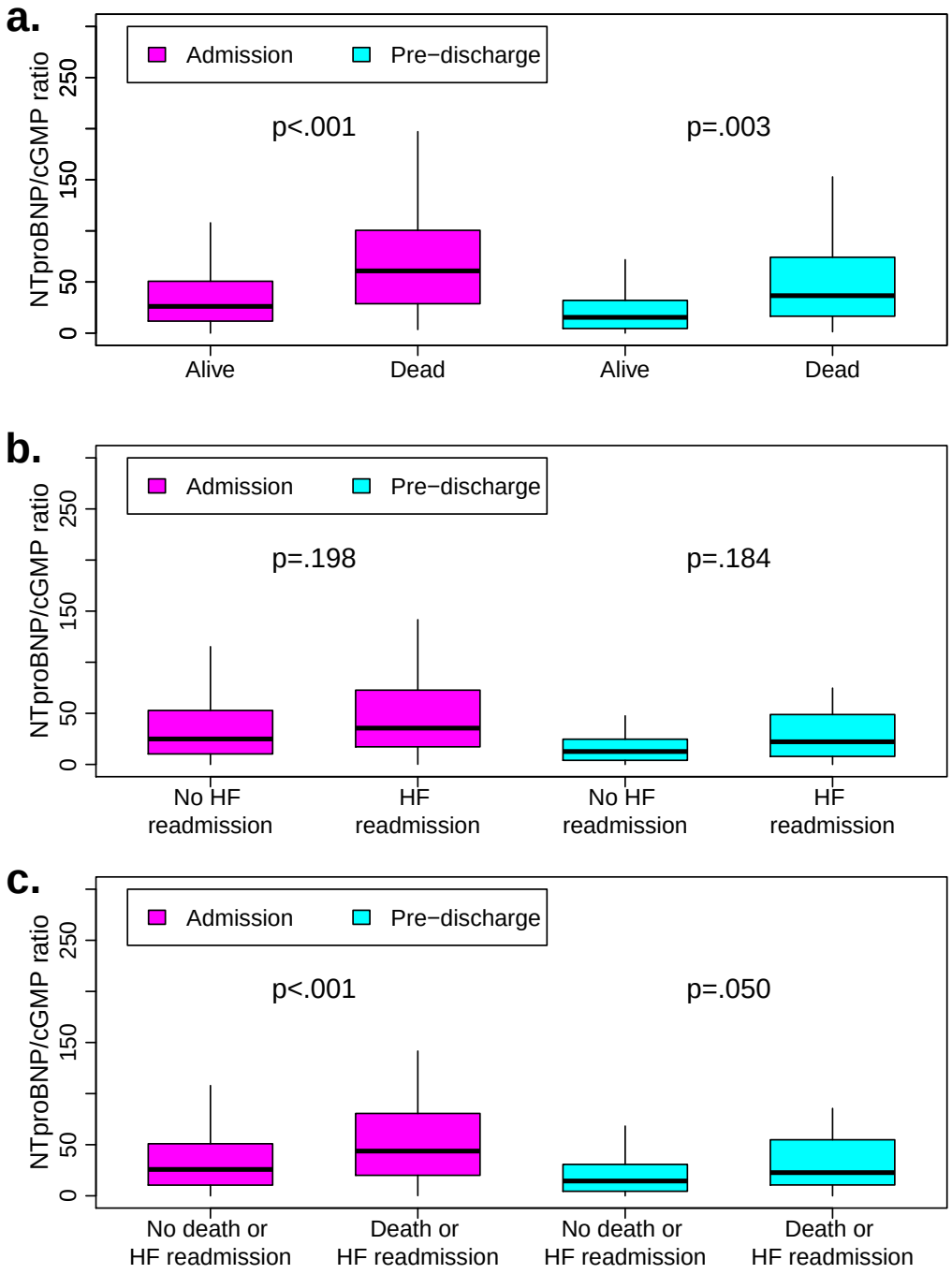

Figure 4.42 Comparison of NTproBNP/cGMP ratio at admission and pre-discharge for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values are shown for the t test.

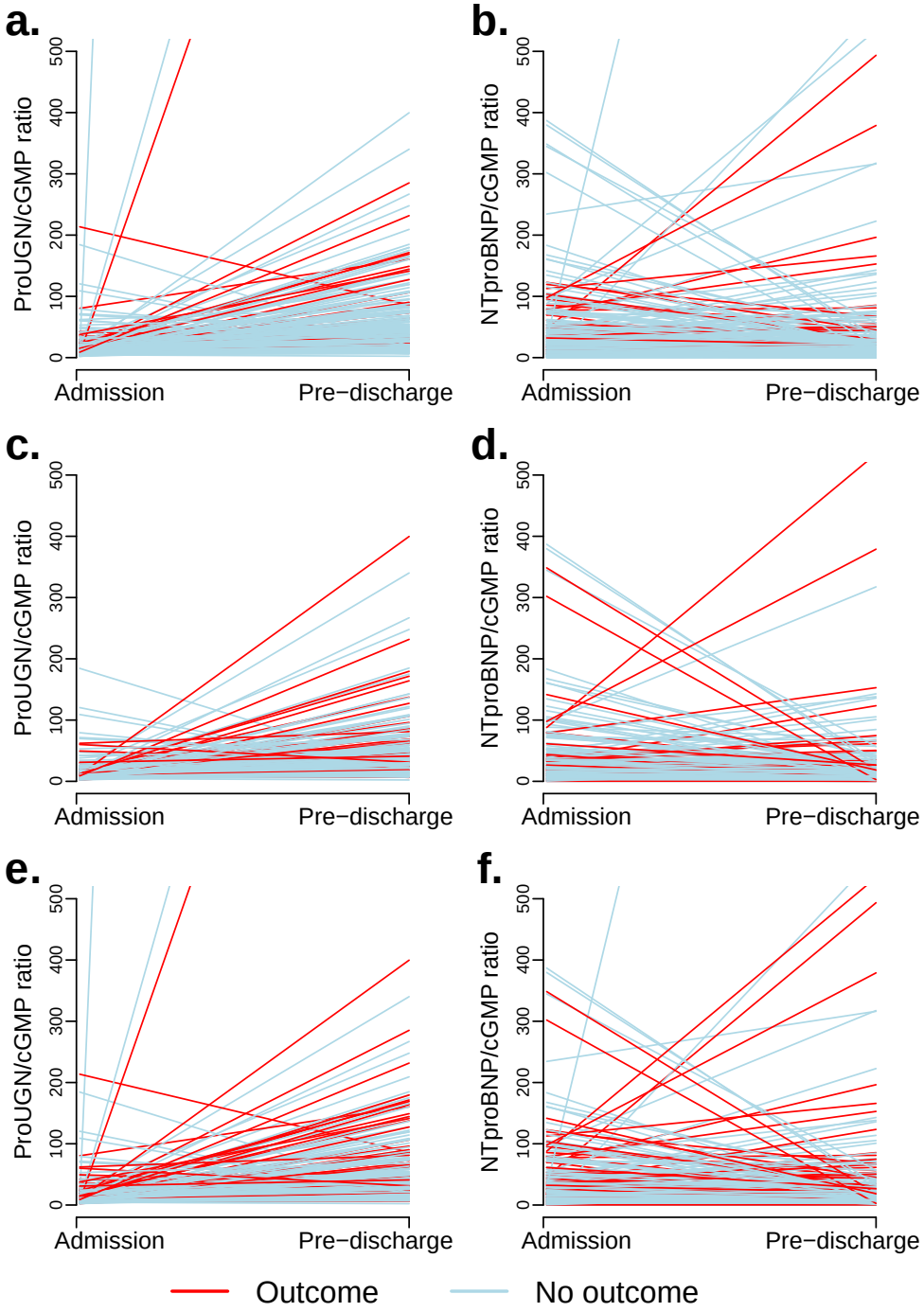

Figure 4.43 Line plots showing individual changes in ProUGN and NTproBNP to cGMP ratio at admission and pre-discharge colour coded for the outcomes: (**a** & **b**) All cause mortality (**c** & **d**) HF readmission (**e** & **f**) All cause mortality or HF readmission. Each line represents a single person.

4.7.2 Logistic regression

The ProUGN pre-discharge/admission ratio was not significantly associated with increased risk of all cause mortality (OR=1.21 [0.40 to 3.70], p=.737), all cause mortality or HF readmission (OR=1.56 [0.67 to 3.71], p=.306) or HF readmission alone (OR=1.96 [0.66 to 6.06], p=.233) in univariate analysis. The cGMP pre-discharge/admission ratio was not significantly associated with increased risk of all cause mortality (OR=0.71 [0.32 to 1.64], p=.400), HF readmission (OR=0.37 [0.12 to 1.00], p=.058) or the combined outcome (OR=0.78 [0.41 to 1.50], p=.449). The NTproBNP pre-discharge/admission ratio was not significantly associated with a greater risk of all cause mortality (OR=2.12 [0.74 to 6.36], p=.170) or HF readmission (OR=0.78 [0.32 to 1.96], p=.581) or the combined endpoint (OR=1.02 [0.47 to 2.28], p=.966) in univariate analysis.

4.7.3 Cox hazards regression

The ProUGN pre-discharge/admission ratio was not significantly associated with early all cause mortality (HR=1.17 [0.42 to 3.29], p=.765), early HF readmission (HR=1.85 [0.70 to 4.88], p=.213) or early all cause mortality or HF readmission (HR=1.46 [0.71 to 3.01], p=.300) in univariate Cox hazards regression. The cGMP pre-discharge/admission ratio was significantly associated with early HF readmission (HR=0.38 [0.15 to 0.99], p=.047) but not with early all cause mortality (HR=0.75 [0.36 to 1.53], p=.425) or the combined outcome (HR=0.83 [0.49 to 1.40], p=.484) in univariate analysis. The NTproBNP pre-discharge/admission ratio was not significantly associated with early mortality (HR=1.96 [0.78 to 4.93], p=.152), early HF readmission (HR=0.74 [0.32 to 1.71], p=.486) or the combined outcome (HR=0.98 [0.48 to 1.99], p=.957) in univariate analysis.

4.7.4 Kaplan-Meier analysis

Kaplan-Meier plots stratified by median ProUGN, NTproBNP and cGMP pre-discharge/admission ratio for outcomes of all cause mortality, HF readmission

and all cause mortality or HF readmission are shown in Figures 4.44, 4.45 and 4.46. A lower NTproBNP pre-discharge/admission ratio was significantly associated with worse outcomes of all cause mortality and the combined endpoint but not HF readmission alone. A lower cGMP pre-discharge/admission ratio was significantly associated with a worse outcome of HF readmission. The ProUGN pre-discharge/admission ratio was not significantly associated with event free survival for any outcome.

4.7.5 ROC curve analysis

Classification by outcome for ProUGN, NTproBNP and cGMP pre-discharge/admission ratio is shown using ROC curves in Figure 4.47. A higher pre-discharge/admission NTproBNP ratio (AUC=.619 [.505 to .734]) was significantly better than the cGMP pre-discharge/admission ratio (AUC=.453 [.328 to .578], p=.024) but not the ProUGN pre-discharge/admission ratio (AUC=.523 [.405 to .642], p=.245) for classification of outcome all cause mortality (Figure 4.47a). For outcome readmission with HF, the pre-discharge/admission NTproBNP ratio (AUC=.538 [.427 to .649]) was not significantly better than the pre-discharge/admission ProUGN ratio (AUC=.577 [.473 to .680], p=.704) or cGMP pre-discharge/admission ratio (inverse AUC=.620 [.510 to .730], p=.210) (Figure 4.47b). For the combined outcome of all cause mortality or HF readmission, pre-discharge/admission NTproBNP ratio (AUC=.570 [.479 to .660]) was not significantly different from pre-discharge/admission ProUGN ratio (AUC=.550 [.462 to .637], p=.722) or pre-discharge/admission cGMP ratio (inverse AUC=.572 [.481 to .663], p=.978) (Figure 4.47c).

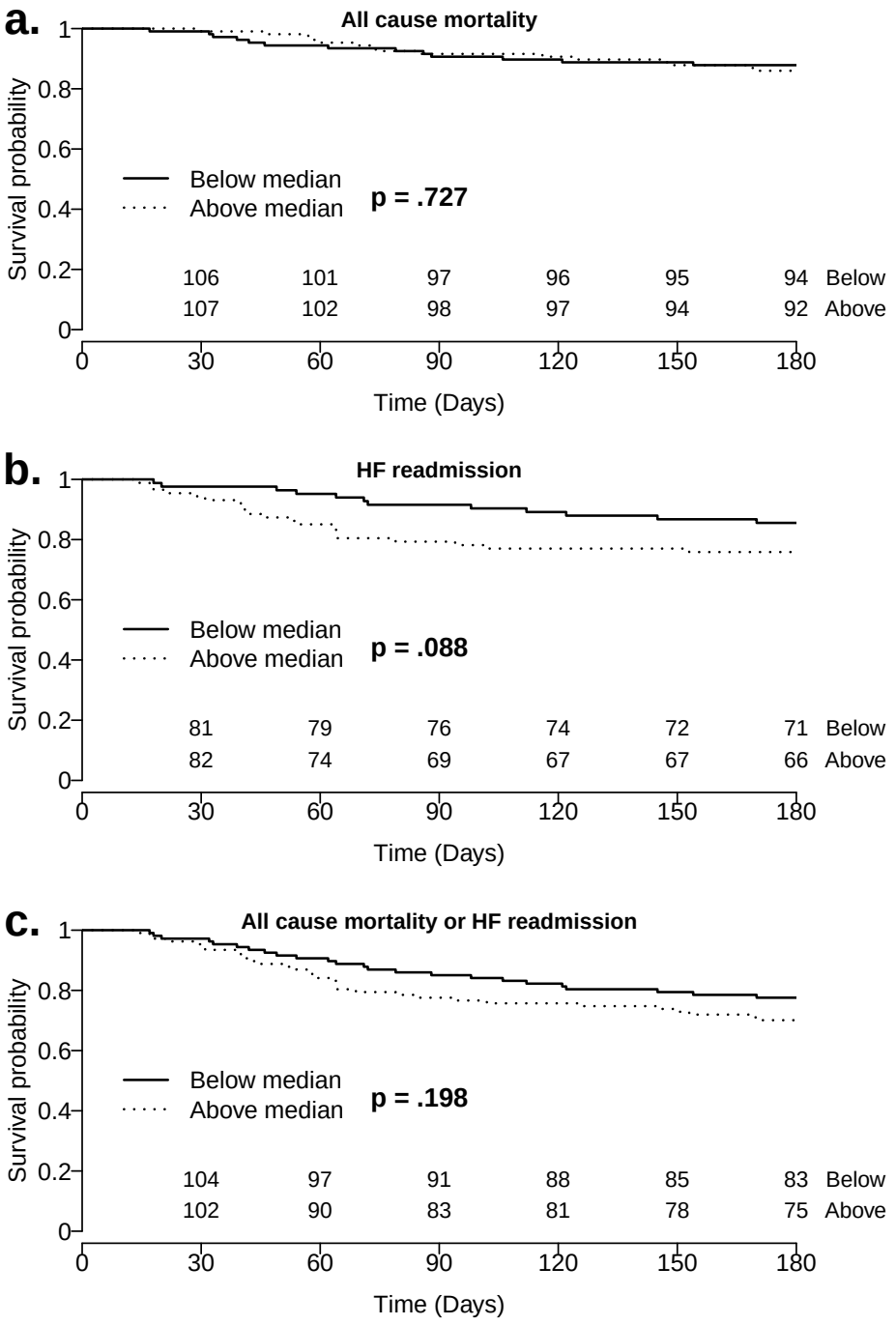

Figure 4.44 Kaplan-Meier plots stratified by median ProUGN pre-discharge/admission ratio for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

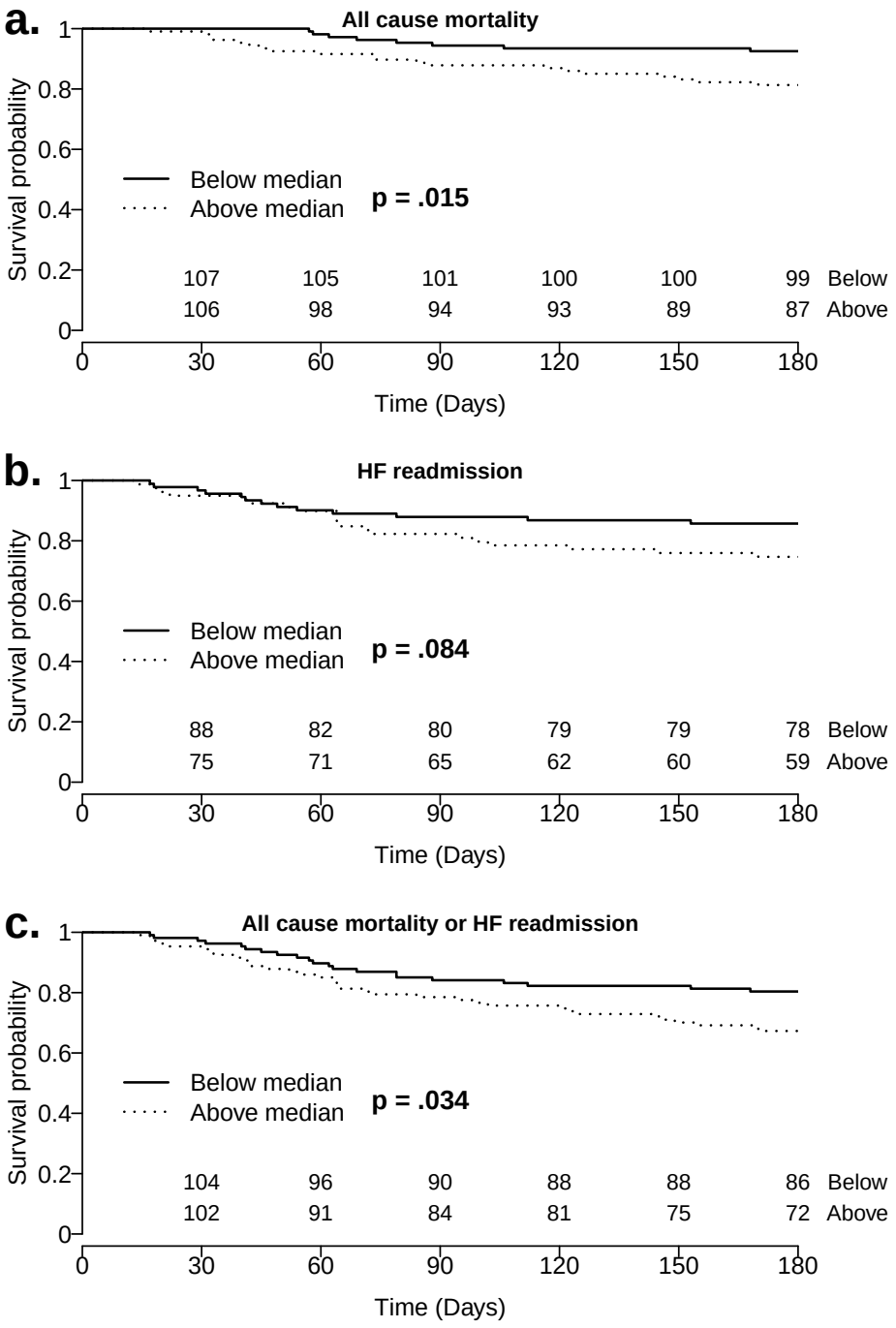

Figure 4.45 Kaplan-Meier plots stratified by median NTproBNP pre-discharge/admission ratio for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

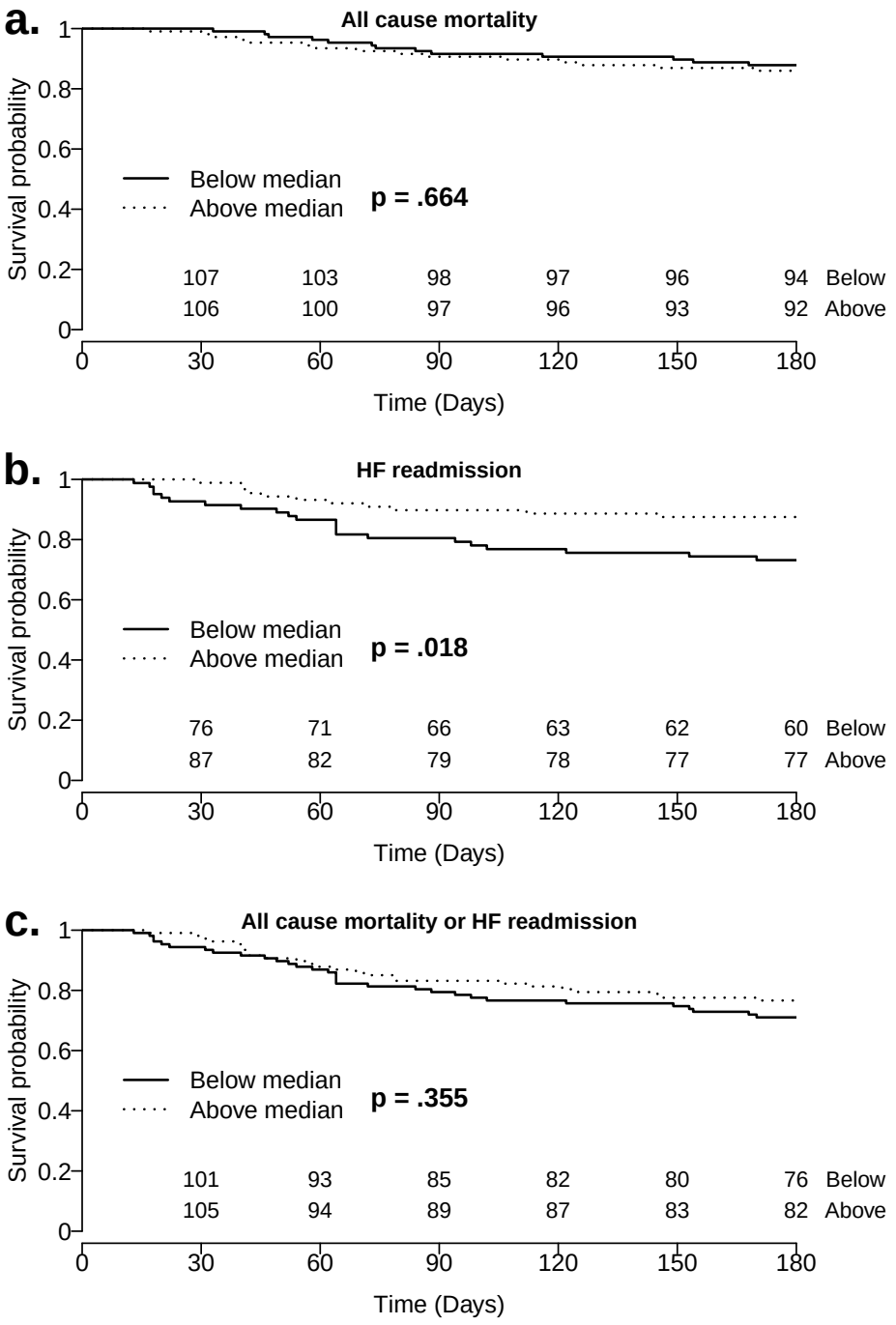

Figure 4.46 Kaplan-Meier plots stratified by median cGMP pre-discharge/admission for outcomes **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. p values for log rank tests are shown next to the legend and numbers at risk are shown at the bottom of each graph.

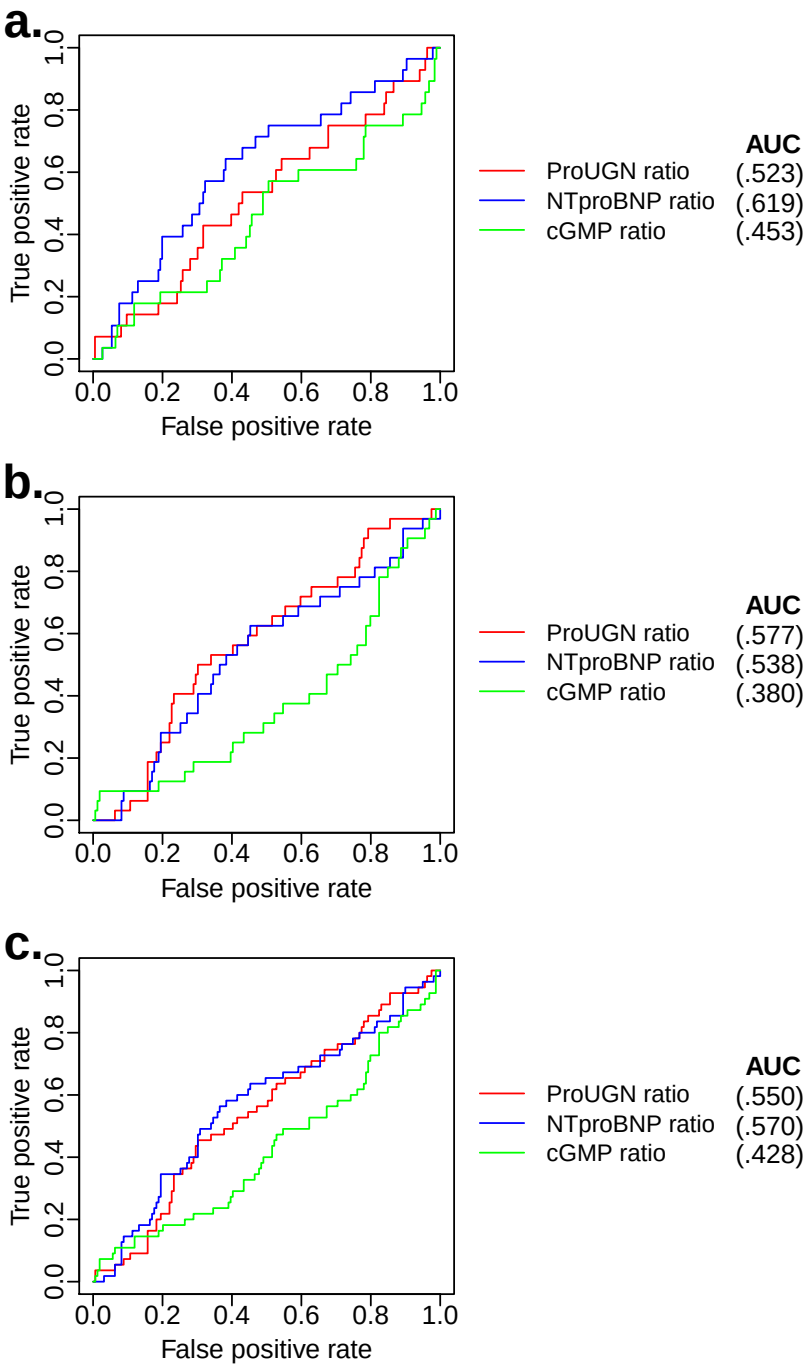

Figure 4.47 ROC curves for pre-discharge/admission ratios of ProUGN, NTproBNP and cGMP for endpoints **a.** All cause mortality **b.** HF readmission **c.** All cause mortality or HF readmission. AUC values are shown in brackets.

Chapter 5

Discussion

5.1 Summary of main findings

The aim of this study was to investigate the role of guanylin peptides in acute and chronic HF. The hypothesis was that dysregulation of guanylin peptides occurs in HF. This was tested by measuring ProUGN, ProGN and cGMP, an intracellular mediator of guanylin peptide activity, in patients with chronic HF and in patients admitted to hospital with acute decompensated HF.

5.1.1 Chronic HF

In patients with chronic HF, plasma ProUGN and ProGN were significantly elevated compared to healthy controls, with higher ProUGN and ProGN levels associated with increasing severity of HF as measured by NYHA class. ProGN and ProUGN levels were closely correlated with each other in both healthy controls and patients with chronic HF. In patients with chronic HF, ProUGN and ProGN levels were significantly greater in those with a history of hypertension, diabetes and those taking diuretics. ProUGN and ProGN were not correlated with measures of cardiac size, but ProGN was positively correlated with EF, while ProUGN was not. Both ProUGN and ProGN were significantly inversely correlated with eGFR. In multivariate analysis, eGFR was the only independent determinant of plasma ProUGN and ProGN level.

5.1.2 Acute HF

In patients with acute HF, admission plasma ProUGN levels were significantly lower than in healthy controls, but by discharge were significantly greater than the admission levels as well as the levels in healthy controls. Admission ProUGN levels were not significantly associated with measures of systolic and diastolic HF severity or cardiac size, demographic factors and past medical history or previous medication history. Admission ProUGN levels were not significantly associated with worse outcomes in linear, logistic and Cox hazards analysis. The accuracy of admission plasma ProUGN level was inferior to NTproBNP for classification of individuals by future adverse outcome as assessed by ROC curves.

Pre-discharge ProUGN levels were significantly greater in those taking betablockers, diuretics and ACEi/ARBs at discharge but not associated with measures of systolic and diastolic dysfunction or cardiac size. Pre-discharge ProUGN was significantly greater in those that died and those that were either readmitted with HF or died compared to those that did not, with higher levels being associated with increased risk of all cause mortality and all cause mortality or HF readmission in univariate logistic regression and all cause mortality or HF readmission in univariate Cox hazards analysis. Pre-discharge ProUGN levels were inferior to pre-discharge NTproBNP levels in classification of individuals by future adverse outcomes. The magnitude of change in ProUGN levels between admission and pre-discharge as measured by the pre-discharge/admission ratio was not significantly associated with patient demographic characteristics, treatment or increased risk of adverse outcomes.

Admission cGMP levels in acute HF patients were were reduced compared to healthy controls, but were greater at pre-discharge than admission, but not significantly different from the controls. cGMP levels at admission showed no significant associations with baseline demographics or medications and showed no significant associations with adverse outcomes in linear, logistic and Cox hazards analysis.

In contrast pre-discharge cGMP levels were significantly lower in those readmitted with HF compared to those who were not as well as higher lev-

els being significantly associated with a reduced risk of HF readmission and early HF readmission in univariate logistic and Cox hazards analysis. The pre-discharge/admission cGMP ratio was not significantly associated with patient demographic factors, past medical history and medications although low ratios showed a non significant trend towards adverse outcomes in linear analysis. A low pre-discharge/admission ratio was significantly associated with higher risk of early HF readmission in univariate Cox hazards regression and Kaplan-Meier analysis.

Admission and pre-discharge ProUGN levels did not correlate with admission and pre-discharge cGMP levels and neither did the change in ProUGN levels between admission and pre-discharge correlate with the change in cGMP levels as measured by pre-discharge/admission ratios. There was no significantly different relationship between ProUGN and cGMP levels at admission and pre-discharge when divided by outcome of all cause mortality and HF readmission.

A greater admission ProUGN/cGMP ratio was associated with higher risk of all cause mortality and early all cause mortality in univariate logistic regression and Cox hazards regression, while a greater pre-discharge ProUGN/cGMP ratio was associated with all adverse outcomes in both logistic and Cox hazards regression. The pre-discharge ProUGN/cGMP ratio was significantly greater in those that experienced all adverse outcomes, while the NTproBNP/cGMP ratio was significantly greater in those who died and who died or had a HF readmission.

5.2 ProUGN in chronic HF

The close correlation between ProUGN and ProGN in both healthy controls and patients with chronic HF supports previous findings that they share many aspects of their physiology, with similar sites of synthesis, stimuli for release, mechanisms of degradation and excretion. However it has previously been demonstrated that plasma UGN has a greater natriuretic effect than GN, likely due to intratubular degradation of GN by peptidases [146]. The recent discovery of a role for ProUGN in appetite regulation [160] raised the possibility

that plasma ProGN may play a role in cardiac cachexia and have other as yet undetermined physiological roles.

Patients with chronic HF had elevated ProUGN and ProGN levels compared to healthy controls when differences in age and eGFR between controls and chronic HF patients were taken into account. As this was an observational study, the direction of this relationship cannot be inferred and the effect of unmeasured variables such as other natriuretic peptides cannot be excluded. Thus elevated ProGN and ProUGN levels may predispose individuals to develop chronic HF, or the development and treatment of chronic HF may lead to a rise in plasma ProUGN and ProGN levels.

In the first case, there may be wide natural variation in ProUGN and ProGN mRNA expression, peptide synthesis and release. As these peptides have both a natriuretic and diuretic effect, it might be expected that those with lower rather than higher levels would be at greater risk of developing HF, as this would lead to a reduced ability to excrete sodium and water, which is observed in chronic HF. ANP and BNP which also act via intracellular cGMP are have cardioprotective effects [184], making it unlikely that increased amounts of ProUGN and ProGN in plasma could cause heart damage or impair renal sodium excretion, although this could be investigated further.

In the second case, the development of HF may result in increased synthesis or release of ProUGN and ProGN, reduced conversion into UGN or GN, or reduced breakdown or clearance. Plasma UGN and GN levels were not measured, so whether there was reduced conversion into their respective active forms, thus increasing the concentrations of their precursors cannot be determined. Increased cellular release of ProUGN and ProGN without upregulated synthesis is unlikely to occur as intracellular storage of ProUGN and ProGN as occurs with ANP has not been observed, as well as the short plasma half life of these peptides [164].

The major sites of synthesis of ProGN and ProUGN are the gastrointestinal tract and renal tubules [125]. Chronic HF is associated with alterations in the gastrointestinal mucosal microenvironment as a result of hypoperfusion and ischaemia, which could upregulate ProGN and ProUGN synthesis or reduce conversion into their biologically active forms [185]. Chronically increased

oral sodium intake upregulates UGN mRNA expression in intestinal and renal tissue in mice and rats [120, 156, 158], although patients with HF are advised to reduce their salt intake.

ProUGN and ProGN levels were significantly greater in patients with a history of hypertension, AF, diabetes and in those taking diuretics. ProUGN but not ProGN was positively associated with EF. Neither ProUGN or ProGN were positively associated with abnormal cardiac structure as measured by LVIDD and LVIDS. This supports the finding that ProUGN and ProGN are not synthesised or stored in cardiac muscle or that their release is dependent on cardiac stretch [104, 125]. Hypertension is associated with impaired renal sodium excretion [186], but the opposite would be expected in the presence of excess plasma ProUGN and ProGN. A history of hypertension, AF, diabetes and taking diuretics were all non significant factors in multivariate linear regression, with only eGFR remaining independently associated with ProUGN and ProGN. Thus renal function appears to account for the greatest portion of variation in ProUGN and ProGN levels when these other variables are taken into account. This supports previous experimental findings that plasma ProUGN is rapidly cleared by the kidney [163].

Despite increased plasma levels of ProUGN and ProGN, patients with chronic HF have impaired sodium and water excretion. Individual urinary sodium concentration was not measured in this study, so the relationship between plasma ProUGN and ProGN and sodium excretion in this cohort cannot be determined. The relative importance of ProUGN and ProGN in renal sodium excretion as compared to other natriuretic peptides and renin-angiotensin-aldosterone activity is also not known, so it is possible they may only play a minor role.

There are several potential explanations for the apparent lack of natriuretic effect of elevated ProUGN and ProGN levels in chronic HF, similar to as previously discussed with other natriuretic peptides in section 1.1.7. These can be divided into mechanisms affecting ProUGN and ProGN activity before their receptors, problems occurring at the level of interaction with their receptors, impaired intracellular signalling following ligand-receptor binding and activation of counter-regulatory mechanisms.

Immunoreactive ProUGN and ProGN detected by the assay may be non functional, either by being the less biologically active stereoisomer or defective. This phenomena occurs with BNP in chronic HF [55, 56], and could be investigated using western blotting, mass spectrometry or NMR to determine the molecular weights and structural conformation of ProUGN and ProGN in HF. Reduced delivery of ProGN and ProUGN to their active sites within the renal tubules may also contribute to reduced natriuresis, as patients with chronic HF had significantly worse renal function compared to healthy controls. Increased degradation of UGN and GN within the tubules by peptidases could act to reduce their natriuretic activity, as chronic HF is associated with increased expression of renal neutral endopeptidases [58]. Pre-treatment with a chymotrypsin inhibitor has been shown to increase sodium excretion in response to guanylin infusions in an isolated perfused rat assay [162], although this converts ProUGN into UGN.

A reduction in expression of renal tubular GC-C receptors or alterations in receptor function are further potential mechanisms which could explain impaired natriuresis despite elevated levels of plasma ProUGN in patients with HF. Renal GC-C receptor expression and activity in HF has not been studied, although as previously noted the GC-C receptor does not seem to be expressed in healthy rat kidney [99]. The identity and activity of ProUGN and UGN receptors in the renal tubules and whether their regulation is altered in HF remains to be determined. Reduced renal expression of GC-A (NPR-A) receptors, receptors for ANP and BNP, have been found in mice with chronic HF, so reduced GC-C receptor expression in humans remains a possibility [59]. Prolonged exposure of T84 cells to STa *in vitro* results in reduced accumulation of cGMP, likely as a result of receptor desensitization and increased PDE activity [187].

GC-C receptor activity is modulated by phosphorylation which upregulates GC-C receptor activity by 70% [101], glycosylation which is correlated with receptor internalisation and desensitization [188, 189], ATP binding [190] and regulation of GC-C gene transcription by PKC [191]. The potential influence of these mechanisms on GC-C receptor activity in chronic HF is yet to be studied. Upregulation of clearance receptors is a further potential mechanism for ProUGN hyporesponsiveness in chronic HF [102], and remains to be investigated.

A reduction in GC-C receptor response may be due to upregulation of PDE [187], and this possibility is discussed later in section 5.4 in the acute HF cohort where plasma cGMP levels were measured.

Enhanced activity of the renin-angiotensin-aldosterone system may act to counteract the natriuretic activity of elevated levels of ProUGN in chronic HF. This has been suggested as a cause for attenuation of natriuresis in response to ANP in chronic HF, as administration of ACEi and ARBs boosts natriuresis in response to ANP in rats with chronic HF [192, 193]. A further possibility is reduced sodium delivery to the renal tubules resulting in impaired natriuresis despite increased levels of ProUGN. This mechanism is thought to contribute to reduced natriuresis in the presence of elevated BNP in HF. Increasing sodium delivery to the distal tubules appears to reverse this phenomena and restore the natriuretic activity of ANP [194].

In summary, plasma ProUGN and ProGN levels are abnormal in patients with chronic HF compared to healthy controls, although whether this is a contributing factor or a consequence of disturbed physiology in chronic HF cannot to be determined. The longer term pathological significance of plasma ProUGN levels was examined in the next part of the study.

5.3 ProUGN in acute HF

Plasma ProUGN levels in patients presenting to hospital with acute HF were significantly lower compared to a cohort of healthy controls who did not differ significantly in age, eGFR and proportion of males. This is in contrast to ProUGN levels in patients with chronic HF which were found to be on average greater than in a healthy control group. ProUGN levels measured in the healthy control groups in the chronic and acute HF arms of the study were most likely different as a result of a new assay being used in the acute HF cohort, thus the measurements of ProUGN levels cannot be directly compared.

These results differ from the findings in a previous study in which ProUGN levels were higher in patients admitted to hospital with acute dyspnoea diagnosed with acute HF compared to in patients with other causes of breathless-

ness [195]. However the comparison group were not healthy controls, so this study is not directly comparable.

ProUGN levels were lower at admission compared to at pre-discharge and in the healthy controls. This suggests that HF patients with chronically low ProUGN levels may be at greater risk of decompensation and thus admission to hospital, or that a fall in ProUGN levels when previously higher is associated with decompensation. The first explanation is less likely as greater not lower admission ProUGN levels were associated with increased risk of all cause mortality and readmission with HF in logistic regression, Cox hazards and Kaplan-Meier analysis, although these associations were non significant. Similarly greater not lower ProUGN levels at pre-discharge were significantly associated with increased risk of mortality and all cause mortality or HF readmission in logistic regression, and all cause mortality or HF readmission in Cox hazards analysis. Furthermore a reduced pre-discharge/admission ProUGN ratio was not associated with all cause mortality, HF readmission or either outcome as might be expected if relatively low ProUGN levels at pre-discharge predisposed to further adverse outcomes. Thus the latter explanation is more plausible as by discharge, ProUGN levels were on a average significantly greater than at admission and in the controls, the situation observed in chronic HF.

A decline in plasma ProUGN levels from previously higher levels could lead to reduced natriuresis and thus sodium and water accumulation, precipitating admission with decompensated HF. Weight gain in patients with chronic HF has been associated with hospital admission with decompensated HF [67]. The process that could trigger a fall in plasma ProUGN when previously stable is unclear. ProUGN at admission was found to be inversely correlated with eGFR as was found in the chronic HF cohort, although this would infer that an increase in renal clearance (a high eGFR) leading to a reduced plasma ProUGN level was responsible. However a higher eGFR at admission and pre-discharge was associated with a reduced risk of all cause mortality and HF readmission in univariate logistic regression and Cox hazards regression making this mechanism unlikely. In addition there were no significant associations between past medical history and ProUGN levels at admission and pre-discharge, thus variation in patient risk factors for cardiovascular disease cannot account

for variation in ProUGN levels either.

Following admission with acute HF, plasma ProUGN levels on average rose during the inpatient stay, so by discharge they were on average significantly greater. As before, since this was an observational study it cannot be inferred that the change in ProUGN levels between admission and pre-discharge leads to recovery or is as a consequence of recovery from acute decompensated HF.

The first hypothesis is initially more attractive, as a greater plasma ProUGN level could contribute to increased natriuresis and diuresis, thus relieving the signs and symptoms of acute HF. The observation of a significant decline in patient weight between admission and recovery, indicating increased water and sodium loss, would also seem to support this hypothesis.

The mechanisms whereby ProUGN synthesis or release increases or breakdown and clearance reduces during recovery from acute HF is uncertain, in particular as to whether this occurs as a result of drug treatments or is related to a physiological or endocrine response. The major source of circulating ProUGN is the gastrointestinal tract [163], and as previously discussed alterations in gut perfusion in HF could influence its release [185].

Plasma ProUGN is mostly converted into UGN in the kidney [164], making it unlikely that reduced conversion of ProUGN into UGN can account for the observed rise in plasma ProUGN. ProUGN levels were not related to measures of heart size or function as measured by LVIDS, EF and mean E/é, so alterations in cardiac wall stretch do not appear to be the stimulus for increased synthesis and release. At admission there were no strong associations between medications and ProUGN levels, while at recovery ProUGN levels were significantly greater in patients on diuretics, betablockers and ACEi/ARBs. How these treatments could increase ProUGN synthesis and release or reduce breakdown is unknown. Loop diuretics are known to impair renal function and thus could reduced ProUGN clearance, although no overall significant difference in eGFR was observed between admission and pre-discharge. However eGFR may not accurately reflect acute changes in renal function in acutely unwell hospitalised patients [196]. At admission ProUGN inversely correlated with eGFR, as is the case in chronic HF, but not at pre-discharge. Neurohumoral activation associated with acute HF independent of treatments could also potentially reduce renal filtration,

increasing plasma ProUGN levels [62].

The second hypothesis is that ProUGN levels rise as a consequence of recovery. A mechanism how recovery from acute HF could stimulate increased ProUGN synthesis and release is unclear. Dietary sodium intake is postulated to increase plasma ProUGN levels, but most patients are placed on low sodium diets during admission, although the frequency and rigour in application of this was not recorded in this study. Although there was a significant fall in weight between admission and pre-discharge, there was no significant difference in plasma sodium, thus haemoconcentration is unlikely to account for the rise in plasma ProUGN. Gastrointestinal release of ProGN may be mediated by cholinergic stimulation of the mucosa, suggesting that an increase in parasympathetic activity during recovery may be a contributing factor [113]. Recumbent posture is associated with decreased sympathetic and increased parasympathetic activity in HF patients [197], while bed rest is associated with increased diuresis and weight loss in HF patients undergoing inpatient treatment [198]. This potential mechanism for explaining variation in ProUGN levels requires further investigation.

5.4 cGMP in acute HF

A previous study of patients admitted to hospital with worsening HF which showed plasma cGMP levels fell between admission and recovery [199], although this was a small study in only 25 patients. Another study showed plasma cGMP levels in patients with acute HF were significantly greater than in healthy controls, and declined significantly over several days although persistently remaining at a higher level than the controls over 7 days [200], although again this was only measured in 4 patients. Urinary cGMP levels have previously been shown to be elevated in chronic HF [201] and a further study in patients with chronic HF showed that higher rather than lower plasma cGMP levels were associated with increased mortality [202], in contrast to the data presented here. Thus the results presented here conflict with previous published evidence of cGMP in acute and chronic HF, and may be as a consequence of a much

greater sample size or different population characteristics.

cGMP is the second messenger for the membrane bound GC receptor family, comprising GC-A (NPR-A) and GC-B (NPR-B) which are receptors for ANP and BNP, GC-C which is a receptor for GN and UGN, as well as for the soluble GC receptor, which is activated by NO [60]. Urinary [203] and plasma [204] cGMP concentrations are markers of GC receptor activity as cGMP is rapidly released in a dose dependent manner from cells stimulated with ANP [205]. In addition plasma cGMP levels have been shown to rise in a dose dependent manner after exogenous ANP infusions in healthy volunteers [206]. No increase in cGMP has been noted with other hormones including thyroxine, parathyroid hormone, noradrenaline and vasopressin [207]. Thus it has been proposed that plasma cGMP levels can be used as a proxy for NO and natriuretic peptide activity on GC linked receptors.

Increased levels of cGMP could arise through greater stimulation of GC linked receptors by natriuretic peptides or NO, upregulation of GC linked receptor expression or activity and decreased breakdown or clearance of cGMP. Nitrates also stimulate cGMP production via soluble GC. Although a higher proportion of patients was taking nitrates at discharge compared to admission (104 at discharge, 66 at admission), there was no significant difference in plasma cGMP between those on them versus those not at discharge.

The rise in plasma ProUGN levels between admission and discharge may account for the rise in plasma cGMP. However there was no positive correlation between ProUGN and cGMP levels at either admission or pre-discharge, nor did the change in cGMP between admission and pre-discharge correlate with the change in ProUGN levels. The relationship between ProUGN and cGMP levels did not significantly differ when patients were grouped by outcomes of all cause mortality or HF readmission. NTproBNP levels declined during inpatient admission making this unlikely to contribute to the increase in cGMP. In addition NTproBNP levels at admission and pre-discharge did not correlate with cGMP levels respectively, nor the change in NTproBNP levels between admission and pre-discharge correspond to the change in cGMP levels.

These observations cast doubt on any relationship between ProUGN and cGMP levels, as might be expected if the increase in plasma ProUGN was the

cause of the increase in cGMP. A number of possible explanations may account for this. One may the effect of unmeasured factors such as ANP and NO, although they have previously been observed to fall during recovery from acute HF [199]. cGMP was not significantly associated with eGFR at admission but was positively correlated at pre-discharge, so reduced renal clearance cannot account for the rise in cGMP levels, as well as there being no significant change in eGFR between admission and at pre-discharge. Different rates of breakdown between cGMP and ProUGN could also alter the balance between their measured concentrations. Heterogeneity amongst acute HF patients might dilute any clear relationship between ProUGN and cGMP levels, although the relatively large sample size should counteract this effect. A final explanation may be that the lack of correlation between ProUGN and NTproBNP levels and cGMP at both admission and pre-discharge indicates impaired activity at GC linked receptors or reduced expression and function of GC linked receptors in patients with HF, as discussed earlier in section 5.2. Thus the lack of a relationship may be pathophysiologically significant in patients with HF.

As well as factors affecting GC-C receptor expression and function, variation in the activity of PDE, the enzyme responsible for the degradation of cGMP of which PDE5 is the most widely expressed in vascular endothelia, may be an important in HF [184]. PDE5 activity is significantly increased in whole kidney and inner medullary collecting ducts in dogs with HF, with inhibitors of PDE5 potentiating the natriuretic activity of exogenous BNP in dog with HF [208]. Thus upregulation of PDE5 would tend to lower cGMP levels in relation to natriuretic peptide concentrations and may contribute to renal hyporesponsiveness to natriuretic peptide action. This could explain the observation that greater ProUGN/cGMP and NTproBNP ratios at pre-discharge were significantly associated with worse outcomes. Previous studies have also shown a blunted response to natriuretic peptides in HF, with chronic HF associated with an increased ANP/cGMP and BNP/cGMP ratios, with a greater ratio associated with worse outcomes [207, 209, 210].

In summary, decompensated HF is associated with relatively low levels of ProUGN and cGMP compared to healthy controls, but high levels of NTproBNP. On recovery, NTproBNP levels fall, but ProUGN and cGMP levels rise. A failure

for cGMP levels to rise in proportion to increased ProUGN levels, as measured by an increased ProUGN/cGMP ratio is strongly associated with worse outcomes. This may indicate that hyporesponsiveness to ProUGN in HF is associated with a poorer prognosis.

5.5 Theoretical and practical implications

This study has shown that elevated levels of plasma cGMP in relation to ProUGN and NTproBNP levels are associated with better outcomes in acute HF. The poor classification utility of ProUGN compared to NTproBNP means that plasma ProUGN is a poor biomarker for adverse outcomes. Natriuretic peptides acting via cGMP have well described cardioprotective effects and are the basis of novel therapies such as synthetic BNP (nesiritide), chimeric peptides (CD-NP) and phosphodiesterase inhibitors (sildenafil) [184, 211–213]. However none have yet demonstrated improved survival benefit in acute or chronic HF. Thus guanylin peptides or GC-C receptor agonists may offer opportunities for novel therapeutic agents for the treatment of acute and chronic HF.

Both the chronic and acute HF studies failed to identify factors accounting for the variation in ProUGN and cGMP levels apart from eGFR. This may be as a result of the heterogeneity of the HF population and in particular patients admitted with acute HF. Better techniques for phenotyping HF patients may be required in order to establish more definite associations.

5.6 Study limitations

The most important limitation is that this is an observational study from which statistical associations between plasma levels of peptides and cGMP with outcomes has been derived. This limits the inferences that can be drawn, as only experimental manipulation of peptide and cGMP levels either in animal models or human subjects can establish causal relationships. In addition these observations would need to be repeated in further cohorts in different geographical locations in order to take into account variation in populations and hospital

treatments.

Comparison between ProUGN levels between the chronic and acute HF cohorts was not possible as different assays were used due to the loss of manufacture of the first kit. In the acute HF part of the study, patients were recruited on the basis of clinical presentation rather than objective measures of systolic or diastolic cardiac function, and so it is possible for a number of cases to have been misdiagnosed as HF, although the distribution of recruited patient characteristics did suggest that the majority of patients had cardiac dysfunction. Patient discharge summaries did not always list HF as the primary diagnosis at admission. In the acute HF cohort, only patients able and willing to consent were recruited which tends to favour younger patients with less severe presentations of acute HF, so the cohort recruited may not be representative of all patients admitted to hospital with HF. ECHO assessment of systolic and diastolic function was not independently performed and thus may be subject to confirmation bias. ECHO assessment is operator dependent and not wholly objective and is thus subject to inter-observer variability. It was not possible to categorise patients admitted with acute HF into those with *de novo* acute HF as opposed to those admitted with decompensated chronic HF, as previous medical records were not always available. The rates of treatment with diuretics (60%), ACEi/ARBs (59%) and betablockers (55%) in those admitted with acute HF would support the view that the latter category were in the majority. Follow up of the acute HF cohort was conducted using the hospital computer records which did not record causes of death. It is also possible that patients moving away from the Leicester area would have been lost to follow up.

5.7 Further research

The scope for further research ranges from increasing basic scientific understanding at the molecular and receptor level to clinical trials. The most important issue is discovering the identity of renal tubular receptors for ProUGN and UGN in humans as well as the precise nature of the peptides which activate them. Then it would be possible to determine if there are any functional differences

in these receptors in patients with HF. Clinical trials could assess the utility of synthetic ProUGN or UGN as a diuretic in patients with HF. Clinical trials into the use of PDE5 inhibitors in HF are still ongoing. ProUGN or cGMP may also have a potential role for monitoring and guiding therapy in patients with chronic HF, although a meta-analysis of trials using this approach with BNP showed no decrease in all cause hospitalisation [214].

5.8 Conclusions

Plasma guanylin peptide levels are deranged in both acute and chronic HF compared to healthy controls. An impaired physiological response to increased plasma ProUGN levels during recovery from acute HF as measured by ProUGN/cGMP ratio is associated with worse outcomes.

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Appendices

Appendix A

Consent form

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 2541414
Fax: 0116 2585631
Minicom: 0116 2586878

Name of Researcher: Professor LL Ng
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Biochemical Markers involved in the prediction of future cardiac events in Acute Heart Failure.

please initial boxes

1. I confirm that I have read and understand the information sheet dated 15/6/2009. (Version 9) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to have blood tests x 2 / blood tests x 5 and an Echo scan. ☐

4. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to samples of my plasma, DNA and urine being stored for 15 years for research into acute heart failure. ☐

6. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

One copy for patient, one for researcher, and one for hospital notes.

Appendix B

Patient information sheet

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 2541414
Fax: 0116 2585631
Minicom: 0116 2586878

Patient information Sheet

Study title: 'Biochemical Markers involved in the prediction of future heart events in Acute Heart Failure'.
Principal Investigator: Professor Leong L Ng, Professor of Medicine & Therapeutics, University of Leicester, Leicester.
Contact details: 0116 252 3125 / 0116 252 5839

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study, as there is no obligation.

1. What is the purpose of the study?

Heart Failure or "weakened heart muscle causing shortness of breath" is a common problem affecting many individuals. We wish to investigate certain proteins which are released during the course of this problem especially during an 'acute admission' to hospital. We wish to see if we can predict which patients are at risk of further heart damage in the future, so we can address their needs. The study will run for 2 years during which time we will follow your progress (through hospital notes record) to see how you are getting on.

2. Why have I been chosen?

You have been chosen to participate in this study as you have been admitted to hospital with heart failure.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in any way.

4. What will happen to me if I take part?

We will invite you to participate in either providing (a) 2 blood and 2 urine tests or (b) 5 blood tests and 2 urine tests.

For study (a) the 2 blood and urine tests will be performed on 2 days during your hospital stay (on admission and on the day of discharge). On each occasion, we will collect approximately 20ml of blood (approximately 4 teaspoons). You will also have an Echo scan (ultrasound scan) of the heart to look at the function of your heart. It is not painful and is part of the normal routine investigation after an admission with Heart Failure. This scan basically involves putting jelly over your chest and taking the pictures of the heart, and normally lasts for ½ an hour or so. This scan will be done during your initial hospital stay.

For study (b), the blood tests and urine tests will be performed on admission, and on the day of discharge, with 3 further blood tests in between, in order to examine more closely changes in the blood proteins and their response to treatment. The admission and discharge blood samples will be 20 ml blood (approximately 4 teaspoons) and the intervening 3 blood samples will be 10 ml (2 teaspoons).

On the day when blood will be taken, it will be spun in a special machine in the laboratory, by which the blood separates into two portions: one thick red portion which is discarded, and other watery clear portion (called 'plasma'), it is the later portion of the blood which is stored for all subsequent analyses. In addition we will extract DNA (genetic material) from the white cells which settle on top of the red cell layer. Urine samples are stored without any processing.

Most of the plasma, DNA and urine samples will be analysed in the first 18-24 months, but all the plasma, DNA and urine samples will be stored for up to 15 years after the end of the study, for measurements of new cardiovascular plasma markers and to determine DNA changes that may aid diagnosis or prognosis of acute heart failure in future, but these will be stored in a coded form to maintain confidentiality. Ethical approval will be sought for further analyses of stored plasma and urine samples.

The study will run for a total of 2 years; during this time we will follow you up mainly by your hospital notes. You will not be contacted as a part of research, but your standard care will continue once you are discharged from the hospital. If you have a hospital admission (due to heart problem) during this period we would know it, you do not need to inform us. The Cardiovascular Unit is also registered with the Office of National Statistics, which periodically supplies us with information on a patient's progress.

5. What are the possible benefits of taking part?

Although there are unlikely to be any direct benefits to you, the information we get from this study may help us to assess future patients with Heart Failure better and assess their risk of further heart damage.

6. What are the possible disadvantages and risks of taking part?

As this project does not involve you changing the drugs you are taking, the risks of taking part are minimal. However, you may experience some bruising and/or discomfort at the site of the blood test in your arm.

7. What will happen if I don't want to carry on with the study?

If you decide to withdraw from this study, your research doctor will make arrangements for your care to continue.

8. What happens when the research study stops?

At the conclusion of the study, we will be able to assess whether any of the blood proteins that we have measured are of value in estimating risk following a Heart failure. These tests may then be used in future risk assessments in patients admitted with a Heart failure.

9. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints mechanisms would be available to you.

10. Will my taking part in this study be kept confidential?

All information, which is collected, about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognised from it. Also with your permission, your own GP will be notified of your participation in the trial.

11. What will happen to the results of the research study?

The results of this research are likely to be published in 2008-09 in a medical journal. All participants in the study will remain anonymous.

12. Who is organising and funding the research?

The University of Leicester and the British Heart Foundation is funding the research. Your doctor will not be paid for including you in this study.

13. Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead, and this study has been given favourable ethical opinion for conduct in the NHS by the Derbyshire Research Ethics Committee (REC). Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

14. Contact for Further Information

For further information about the study you may wish to contact:

Prof LL Ng (FRCP. MD)
Professor of pharmacology & Therapeutics
University of Leicester
Level 4, Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Infirmary Road
Leicester, LE2 7LX
Tel: 0116 252 3125 / 0116 252 5839

In case of concern or complaint, please contact
UHL Complaints Office
Gwendolen House,
Leicester General Hospital,
Leicester
Tel : 0116 258 8718

Appendix C

Letter to patient's GP

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 2541414
Fax: 0116 2585631
Minicom: 0116 2586878

Dear Dr

Your patient _____ has kindly agreed to take part in our study entitled "Biochemical Markers involved in the prediction of future cardiac events in Acute Heart Failure." This is a prospective study that hopes to enrol 316 patients to look at the effect of novel cardiac peptides (Arginine Vasopressin, Adrenomedullin, Endothelin-1 etc) to try to predict prognosis in patients with Heart Failure. This study will run for a period of 2 years. As a part of care the patient will be followed up (if required) by the medical team looking after him, but as a part of research we may contact you for follow up purposes.

If you have any further queries about the project then do not hesitate to contact us on the above number.

Yours sincerely

Prof LL Ng (FRCP. MD)
Professor of pharmacology & Therapeutics
University of Leicester
Level 4, Clinical Sciences Building
Leicester Royal Infirmary
Infirmary Road
Leicester, LE2 7LX
Tel: 0116 252 3125 or 252 5839

Appendix D

Case report form

Biomarkers in Heart Failure Patient CRF **Study ID No.** ☐☐☐☐☐

Screening Date ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

Tel No ☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Admission Date ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

Hospital ☐☐☐ Admitting Ward ☐☐☐

Ward of care ☐☐☐☐☐☐☐☐☐☐

Age ☐☐ M ☐F☐ ☐Caucasian ☐South Asian ☐Black ☐Other

☐ **Admission Blood Sample** **Date** ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

☐ **Admission Urine Sample** **Date** ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

☐ **2nd Blood Sample** **Date** ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

☐ **2nd Urine Sample** **Date** ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

Inclusion Symptoms

- ☐ Dyspnoea at rest
- ☐ Dyspnoea on exertion
- ☐ Fatigue
- ☐ Orthopnea
- ☐ PND

Inclusion Signs

- ☐ Oedema
- ☐ ↑ JVP
- ☐ 3rd HS
- ☐ Rales
- ☐ Murmur
- ☐ Displaced Apex

Exclusion Criteria

- ☐ Surgery prior month
- ☐ Life expectancy<6m

Other Symptoms

- ☐ Chest Pain
- ☐ Cough
- ☐ Fever

Main Diagnosis

- ☐ New onset HF
- ☐ Decompensated HF

Other Diagnoses

- ☐ LRTI
- ☐ ACS
- ☐ Pleural Effusion
- ☐ Sepsis
- ☐ Dysrhythmia
- ☐ Exacerbation of COPD

Aetiology of HF

☐ IHD ☐ Hypertension ☐ Valve Disease ☐ Idiopathic DCM ☐ Restrictive ☐ Unknown

☐ Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Examination **Date** ☐☐-☐☐-☐☐ **Time** ☐☐.☐☐

HR ☐☐☐ Temp ☐☐.☐

sBP ☐☐☐ Height ☐☐☐cm

dBP ☐☐☐ Height ft☐In☐☐

RR ☐☐ Weight ☐☐☐.☐

BM ☐☐.☐

NYHA at admission 1☐2☐3☐4☐

NYHA prior to admission 1☐2☐3☐4☐

Killip Score 1☐2☐3☐4☐

CVS Risk Factors

☐DM ☐Hypertension ☐↑Lipids ☐Smoker ☐Ex-Smoker ☐FH IHD ☐BMI>30

Medical History

☐HF ☐Angina ☐MI ☐AF ☐Angio ☐PCI ☐CABG ☐ICD ☐PPM ☐CRT ☐MS

☐RF ☐Asthma ☐PVD ☐CKD ☐CVA ☐TIA ☐COPD ☐PVR ☐Gout ☐AS ☐AR

☐ Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

☐ Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Admission Medication

☐Aspirin ☐Clopidogrel ☐Doxazosin ☐ISMN ☐PPI ☐Sulphonylurea

☐Amiodarone ☐CaBlocker ☐Eplerenone ☐Ipratropium ☐Ranitidine ☐Thyroxine

☐Allopurinol ☐Digoxin ☐Glitazone ☐Metformin ☐Spiro ☐Warfarin

☐BZD ☐Diltiazem ☐Hydralazine ☐Nicorandil ☐Steroids

☐B2 Agonist ☐Dipyridamole ☐Insulin ☐NSAID ☐Statin

☐ Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

☐ Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

☐BetaBlocker Drug ☐☐ Dose ☐☐☐ ☐Frusemide Dose ☐☐☐

☐ACEi Drug ☐☐ Dose ☐☐☐ ☐Bumetanide Dose ☐☐☐

☐ARB Drug ☐☐ Dose ☐☐☐ ☐Metolazone Dose ☐☐☐

Initial Treatment

☐GTN ☐CPAP ☐NIV ☐Ionotropes ☐Cardioversion ☐IABP ☐Antibiotics ☐Insulin SS

☐Frusemide Dose ☐☐☐

Inpatient Management & Events

☐Angio ☐PCI ☐CABG ☐PPM ☐ICD ☐CRT-D ☐Open Valve Repair ☐TAVI

☐Cardiac Arrest ☐VT ☐VF ☐ITU Admission

ECHO **Date** ☐☐-☐☐-☐☐

EF ☐☐ LVIDD ☐.☐☐ LVIDS ☐.☐☐ ESV ☐☐☐ EDV ☐☐☐ WMSI ☐.☐

Medial E/E` ☐☐.☐ Lateral E/E` ☐☐.☐