Molecular forms of Brain Natriuretic Peptide and Cardiotrophin-1 in the assessment of Left Ventricular Systolic Dysfunction after Myocardial Infarction and in subjects with Chronic Heart Failure

A thesis submitted for the degree of Doctor of Medicine at the University of Leicester

by

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July 2000

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Acknowledgments

The completion of this thesis would not have been possible without the support of Dr Leong Ng. His advice, friendship and not least patience have been invaluable over the years both with regards to this thesis and in clinical matters. My appreciation is further extended to Dr Iain Squire who I cannot thank sufficiently highly for his help over the years. I am also grateful to Dr. Joan Davies for her favorable assistance with the clinical studies. My kindest regards are relayed to Professor Barnett for his help. My thanks are also extended to Miss Pauline Quinn, Miss Sonja Jennings and Dr. Louise Hardy for their excellent technical assistance throughout the year. In addition I owe considerable thanks to the patients and normal volunteers who were willing to partake in the studies described.

I acknowledge with gratitude the Leicester Royal Infirmary Fellowship award for the financial support without which this work would not have been possible.

I wish to express my thanks to my parents for all their help and encouragement over many years of study. Their support and love at difficult periods has not been forgotten.

Finally I would like to thank my wife Punita for her enormous support in preparing this manuscript. There were times when she thought I would never come out of my den! This work is dedicated to Punita and to our children Neil and Sara.

Publications obtained whilst doing research on the Molecular forms of Brain Natriuretic Peptide and Cardiotrophin-1.

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INTRODUCTION

Heart failure is common, has a poor prognosis and can be treated. The incidence is increasing, partly because of improved survival after myocardial infarction and partly because the population is aging and heart failure is a disease of the elderly. Despite advances in our understanding of the pathophysiology of heart failure, the diagnosis of heart failure remains enigmatic and requires the skills of the physician. Those skills have not yet been superseded by technology.

The natriuretic peptide family of hormones have been used extensively to study a range of cardiovascular disorders. Plasma levels of brain natriuretic peptide (BNP-32), are elevated following myocardial infarction and in patients with heart failure with well documented associations of BNP-32 with indices of left ventricular function and prognosis in patients suffering these conditions. Amino-terminal pro brain natriuretic peptide (NT-proBNP) is a recently identified processing fragment of preproBNP (γ-BNP) which circulates in plasma in higher concentrations than BNP-32 itself and may represent a more competent marker of underlying left ventricular function. Measurement of plasma NT-proBNP may serve an objective and cost-effective method for investigating ventricular dysfunction in clinical practice.

Another candidate peptide for assessing left ventricular systolic dysfunction is Cardiotrophin -1 (CT-1), a member of the growing family Interleukin-6 (IL-6) related cytokines that function via the glycoprotein 130 signalling pathway. CT-1 was originally identified as a cytokine capable of inducing cardiac hypertrophy. More recent indications from animal work suggest a role for CT-1 in the pathogenesis of left ventricular dysfunction following myocardial infarction and in models of heart failure.

There is a clear need to take a broader view of heart failure management and seek to apply improvements in diagnostic methods and treatment more widely in the community for the benefit of all eligible patients. Increased emphasis on the early detection of left ventricular systolic dysfunction and intervention to delay progression and improve long term outcome should be our main priority.

CHAPTER 1

LEFT VENTRICULAR SYSTOLIC DYSFUNCTION

Heart failure (HF) is not a diagnosis, not even one syndrome, but a cluster of syndromes, each syndrome being of varied etiology. The patient with heart failure is usually elderly, and disease in multiple systems is the rule rather than the exception, requiring a holistic approach to patient care.

Left ventricular systolic dysfunction (LVSD), which can be observed by echocardiography, is the commonest cause of the clinical syndrome of heart failure. HF is defined as symptomatic LVSD [1,2].

In most industrialized countries, HF arises mainly as a consequence of coronary artery disease (CAD), usually following an acute myocardial infarction (AMI) [1,3]. It is known that many patients with significant myocardial damage will go on to develop HF [4,5,6]. These patients often go through a phase of 'asymptomatic left ventricular dysfunction' where objective measurement reveals impaired cardiac contractility but overt heart failure is not present. This latent stage can be readily detected and, if treated, HF and its consequences can possibly be prevented [4].

INCIDENCE AND PREVALENCE

The incidence of HF has been steadily increasing over the past 10-20 years, as have hospital admissions and death caused by the condition [7,8]. These trends are likely to persist because the prevalence of HF rises steeply with age [9] and an increasing proportion of the population is now

living beyond 75 years. The condition currently affects 1% of the population and approximately 10% of those greater than 75 years old [10]. The annual incidence rate of new onset HF is approximately 300/100,000 [11].

Recent data suggest the prevalence of asymptomatic LVSD is also of the order of 1-2% i.e. similar to that of overt HF [1,12].

PROGNOSIS

HF is associated with a high mortality rate, the probability of dying within five years after the onset of heart failure being 62% for men and 48% for women in the Framingham study [13]. More recently, inpatients who have required hospital assessment, one year mortality may be 10-20% in those with 'mild-moderate' symptoms and as high as 40-60% in those with severe symptoms [14,15].

HOSPITAL ADMISSIONS

In the UK, around one third of patients with HF require re-admission within 12 months of the index admission [16]. In a recent review of the increase in emergency admissions in Scotland, HF was one of the four most common reasons for three or more emergency admissions in those aged 65-84 years and the commonest in those greater than 85 years [17]. In light of this it is not surprising that HF is a major economic burden to the health care systems of developed countries, accounting for 1-2% of total health care expenditure [18]. Around 60% of this cost is related to hospitalisation [19].

TREATMENT

Several studies have shown that the prognosis of patients with HF can be improved by treatment with an angiotensin converting enzyme inhibitor (ACEI) and more recently beta blockers (BB) [20,21,22]. This also applies to those patients with asymptomatic LVSD as they too benefit from therapy [4].

In spite of this evidence, a significant proportion of patients with LVSD do not receive this therapy [23]. At least part of this shortfall can be explained by the difficulty in diagnosing LVSD. Many of the signs and symptoms of HF are non-specific; moreover their absence by no means excludes the diagnosis [24]. As a result it has been suggested that all patients with clinically suspected HF or those at risk of asymptomatic LVSD should be investigated to confirm the diagnosis [25]. Echocardiography has become the standard investigation for this purpose, but provision remains limited [25].

CLINICAL DIAGNOSIS OF LVSD

Clinical diagnosis of HF is not difficult in the advanced and severe stages of the condition. However in milder cases the typical symptoms of breathlessness, fatigue and ankle swelling are often non-specific. Such symptoms are often difficult to interpret particularly in the elderly, obese or female patients [26].

The clinical signs of HF although reasonably specific are not at all sensitive. Davie studied the predictive accuracy of clinical signs in hospitalised patients and concluded that a displaced apex beat with a sensitivity of 66% and a specificity of 96% was the best predictor of left ventricular systolic dysfunction [27]. Isolated basal crepitations are a poor predictor of left ventricular systolic dysfunction having a sensitivity as low

as 13% and a specificity of 35% [28]. As such the adequacy of diagnosis of HF is poor, with reported levels of false positive diagnoses in primary care ranging from 30 to 50% [29].

Furthermore, a notable number of patients with significant myocardial damage pass through a period of asymptomatic LVSD before developing overt symptoms. This asymptomatic form of LVSD is clinically undetectable.

Table 1 shows the relative sensitivity and specificity of the common signs and symptoms of HF in predicting the presence of the syndrome, as found in a series of 1300 patients undergoing cardiac catheterisation.

INVESTIGATIONS

The chest-X ray has been shown to be a poor diagnostic tool in detecting the presence or severity of LVSD. There is a poor relationship between heart size and left ventricular (LV) systolic function [30].

There have been contradictory reports with regard to the usefulness of the electrocardiogram (ECG) in HF. It has been shown that LVSD is unlikely to be present if an ECG is normal or shows only minor abnormalities [31]. The ECG has been shown to have a sensitivity of 94% and a specificity of 61% in detecting LVSD [31]. However less promising findings have been demonstrated by Houghton et al where 10% of patients with significant LVSD would be missed if the ECG was to be used as a screening tool for suspected HF [32].

This has therefore led to the suggestion that all patients in whom HF is suspected should have an echocardiogram. However the availability of

echocardiography is restricted in contrast to the number of patients in whom the diagnosis of HF requires confirmation or exclusion.

CONCLUSION

Given the clinical difficulty of clinical diagnosis of LVSD in many cases, diagnostic investigations are of paramount importance in the identification of patients with LVSD. Electrocardiographic and radiological investigations have an important but limited role in this respect. Echocardiography shall remain the gold standard but resources are limited and clearly echocardiography cannot be used to screen all patients in the general population. As such more widely available and alternative diagnostic tests are being considered as aids to the diagnosis of LVSD, in particular Neurohormones and cytokines.

TABLE 1:

	Sensitivity(%)	Specificity (%)	Positive predictive value(%)
Medical history			
Dyspnoea	66	52	23
Orthopnoea	21	81	2
Nocturnal dyspnoea	33	76	26
History of oedema	23	80	22
Physical signs			
Tachycardia	7	99	6
Rales	13	91	27
Oedema	10	93	3
Added heart sounds	31	95	61
Elevated JVP	10	97	2

CHAPTER 2

NEUROHORMONES

Structure & Synthesis

Brain natriuretic peptide (BNP) is a 32 amino acid peptide [33] that is synthesised predominantly in the left ventricle as the 108 amino acid prohormone prepro-BNP (γ-BNP) (Figure 1) [34,35,36]. It is produced in response to an increase in wall stress in the left ventricle [48]. The hormone is a potent vasodilator and natriuretic factor that regulates salt and water homeostasis.

Indicator of LV function and prognosis following an AMI and in subjects with HF

Recently, interest has focused on the use of hormonal markers such as BNP, atrial natriuretic peptide (ANP) and N-terminal pro-ANP (NT-ANP) as indices of LVSD and prognostic indicators of outcome following AMI [37]. In LVSD and following AMI, BNP synthesis is increased in both the infarcted and non-infarcted myocardium [38]. Thus the secretion of this peptide may reflect not just left ventricular dysfunction but may be a more sensitive index of abnormal wall stress that could precede the process of ventricular remodelling [39,40]. Recent studies have indicated that elevated levels of ANP, NT-ANP (the N-terminal fragment of the ANP prohormone) and BNP are powerful predictors of cardiovascular mortality after AMI [37,41]. Of these measures, BNP may have the greatest potential to complement the standard prognostic indicators for risk stratification in patients with LVSD [42].

An experimental study demonstrated increased BNP mRNA expression and secretion in non-infarcted regions of myocardium (primarily in the borderline region between infarcted and non-infarcted regions where wall stress is maximal) [38]. As such, plasma concentrations of BNP may prove a sensitive index of the aberrant wall stress that is a preceding influence towards the development of ventricular remodelling [40] .

Investigation of the time course of plasma BNP-32 secretion following myocardial infarction has demonstrated the development of either a monophasic or biphasic pattern of secretion. Plasma levels of BNP reach an initial peak 16 hours following admission with the emergence of a secondary rise developing in a proportion of patients at 5 days which is more commonly associated with anterior transmural infarction and the development of left ventricular dysfunction [39]. Plasma levels of BNP remain significantly elevated 4 weeks from infarction [39]. The marked initial increase in plasma BNP suggests that raised levels are a result of myocardial necrosis (i.e. discharge of BNP from injured myocytes). However, the second peak may relate to the evolution of infarct expansion (with its associated rise in wall stress), and the associated development of LVD.

Circulating concentrations of BNP are also raised in LVSD and in chronic HF [43,44]. It has been suggested that circulating BNP may be used as a marker of asymptomatic LVSD in patients with suspected or known cardiac disease [45].Two studies have shown that BNP is a good indicator of LVSD in the general population. Cowie et al demonstrated that in patients presenting to a general practitioner with suspected HF, plasma BNP can reliably identify those who merit further investigation [46]. A larger study by McDonagh et al also showed that plasma BNP could be used to screen the

general population for LVSD, especially when the test is targeted at high risk groups [47]. The sensitivity and specificity for BNP identifying LVSD was 76% and 87% respectively which improved to 92% and 72% in patients over 55 years [47]. Although the positive predictive value for the detection of LVD was disappointingly low (16%) it is similar or better than those reported for other screening tests such as the use of prostate specific antigen for the identification of prostate malignancy. The authors concluded in suggesting that BNP could be used to screen the general population for LVSD, particularly when aimed at high risk groups [47].

NT proBNP

However, the standard assays for plasma BNP measure total BNP with no separation of the components into the high molecular weight prepro-BNP form or the lower molecular weight and active BNP-32, even though more detailed studies using gel permeation chromatography of these plasma extracts have clearly demonstrated the presence of these two components [48,49,50].

Recent work has reported the existence of NT-proBNP (a 8.6 kD peptide, the N terminal 76 amino acids of prepro-BNP) and this has been shown to circulate in plasma in higher concentrations than BNP-32 in a small study of patients with HF [51]. NT-proBNP is elevated even in NYHA Stage I HF [52]. The maturation of proBNP into BNP-32 and NT-proBNP is likely to take place prior to release into the circulation [57]. Furthermore, by analogy with the atrial natriuretic peptide hormonal system, where the N-terminal precursor provides a more sensitive index of cardiac dysfunction than ANP itself, it seems judicious to suggest that a similar association exists for the BNP family of peptides. As such NT-proBNP represents a potentially more discerning marker of cardiac impairment than BNP-32. Richards et al also

determined that an NT-BNP level ≥ 160pmol/l had a sensitivity and specificity of 91% and 72% respectively, for the subsequent prediction of 24 month mortality which was superior to BNP-32, ANP, NT-ANP and left ventricular ejection fraction [53].

Clinical implications

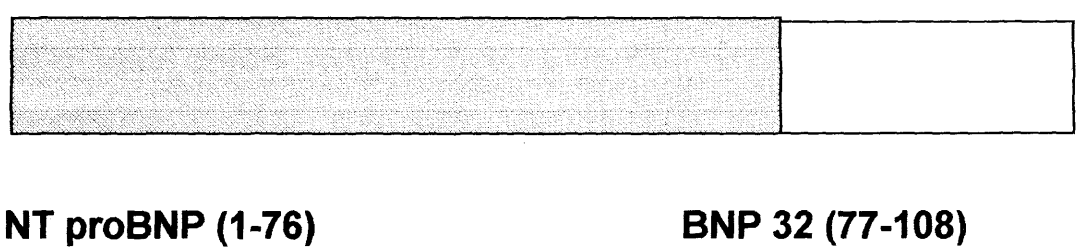
Although the outlook for patients with HF is poor, prognosis can be improved by appropriate therapy with ACE inhibitors. Due to difficulties in diagnosing the condition, many patients with LVD are excluded from therapy. However, the development of plasma assays for hormones that may be elevated in LVSD may lead to non-invasive techniques for assessing ventricular function which could be used to both diagnose LVSD and monitor the response to medical therapy. The natriuretic peptides and their precursors may form the basis of such a non-invasive test. A comparison of techniques for assessing LVSD after AMI suggested that BNP-32 may be as sensitive as echocardiography and superior to clinical examination [54].

Conclusions

The use of the Natriuretic peptides will clearly not replace established methods of assessing ventricular dysfunction such as echocardiography and radionuclide ventriculography. However such investigations (particularly when aimed at high risk individuals) may identify those patients in whom further investigation is required more accurately than on clinical criteria alone. This would limit inappropriate and unnecessary investigations, but moreover would allow for the earlier identification of patients with both symptomatic and asymptomatic LVSD.

The potential impact of population based screening for heart failure is widespread. A blood test measuring a biochemical marker of LVSD is an exciting and challenging prospect. Its precise role in population based screening programs requires further and more detailed investigation.

Figure 1: Structure of the prepro BNP peptide.



CHAPTER 3

CARDIOTROPHIN-1

Cardiotrophin-1 is a member of the growing family of interleukin-6 (IL-6) related cytokines that bind to glycoprotein 130 (gp130) receptors that include interleukin-6, interleukin-11, ciliary neurotrophic factor (CNTF) , leukaemia inhibitory factor (LIF) and oncostatin M [55]. The novel cytokine CT-1 was cloned recently from a mouse embryo cDNA library by expression cloning and was characterised as a cardiac myocyte hypertrophy-inducing factor [55], adding sarcomeres in series rather than in parallel [56] and leading to increased cardiac myocyte size by an increase in cell length with little change in width.

CT-1 binds to a signalling complex consisting of the leukaemia inhibitory factor receptor and glycoprotein 130 (gp130), exhibiting biological activity in cardiac myocytes such as induction of immediate early genes through activation of gp130 [56,57]. An additional component of this signalling complex is a 80 kD protein and the pathway employed to the nucleus is the Janus kinase/signal transducer and activation of transcription 3 (STAT3) pathway [58].

The importance of CT-1 in cardiac development and hypertrophy was established in transgenic models. Transgenic mice that overexpress the IL-6 and IL-6 receptor (hence constitutively activating gp130) demonstrate myocardial hypertrophy [59]. In contrast disruption of the gp130 gene was lethal in mice, resulting in immature hypoplastic ventricular myocardium [60]. CT-1 is also expressed at high levels in the myocardium during the course of cardiogenesis, and promotes the proliferation and survival of embryonic cardiomyocytes [55,56,57].

The IL-6 family of cytokines also exhibit the remarkable property of cytoprotection for cells subject to damaging stimuli. For example, activation of gp130 by LIF initiates expression of cytoprotective genes in cardiac myocytes such as bcl-x [61] and CT-1 prevented apoptosis of myocardial cells via a pathway dependent on activation of a mitogen activated protein kinase (MAPK) [62]. Thus, different responses of the cardiac myocytes to CT-1 result from activation of different kinase cascades; the hypertrophic response of cardiac myocytes to CT-1 employed signalling via the Jak/STAT pathway, whereas the antiapoptotic effect depended on the MEK/MAPK pathway. Further evidence of a cardiac cytoprotective effect was evident in experiments where CT-1 inhibited the production of tumour necrosis factor alpha (TNFα) production in the heart and serum of lipopolysaccharide treated mice [63]. TNFα is a putative factor in the pathogenesis of congestive cardiac failure [64].

The acute haemodynamic effects of CT-1 were also recently documented in rats [65]. CT-1 led to an increase in cardiac output via an increase in heart rate and a reduction in systemic vascular resistance without a direct positive inotropic effect. Together with its cytoprotective effects, it may improve cardiac function via a reduction in afterload.

Whilst the reversion of myocytes following an ischaemic insult to a more primitive phenotype with secretion of CT-1 and BNP (brain natriuretic peptide) may initially be beneficial (since both CT-1 and BNP would reduce afterload, and CT-1 also has additional cytoprotective properties), the continued secretion of CT-1 may conceivably be deleterious, since the eccentric hypertrophy which results from addition of sarcomeres in series may lead to ventricular dilatation and irreversible loss of function (with similarities to the eccentric hypertrophy in volume overload situations). In

addition, CT-1 has been shown to stimulate BNP production at a transcriptional level from cultured neonatal rat cardiomyocytes [66], and raises the possibility that CT-1 is involved in the secretion of BNP clinically observed in severe cardiac hypertrophy. This BNP gene induction takes place via a distinct mechanism when compared to that of endothelin-1 (ET-1) [67].

More recently it has been shown that augmented gene expression of CT-1 and its receptor component may play a central role during ventricular remodelling after myocardial infarction in a rat model [68]. In another animal model, substantial overexpression of CT-1 and gp130 occurs in the heart during experimental acute Chagasic cardiomyopathy [69]. The published work of Gerdes et al on the morphology of cardiac myocytes in ischaemic cardiomyopathy has documented that cells were about 50% longer than normal cardiomyocytes thus suggesting addition of sarcomeres in series in LVD [70]. These animal experiments and the morphometric studies on human cardiomyocytes strongly suggest a role for CT-1 in the ventricular remodelling following acute myocardial infarction in humans and also in patients with established heart failure, but there is at present no data on plasma CT-1 measurement in man.

CHAPTER 4

AIMS OF THE STUDIES

In this project, we propose to :-

1. develop sensitive and specific ILMA (immunoluminometric assay) for measurement of CT-1 and establish the normal range for CT-1.

2. examine the hypothesis that NT-proBNP and CT-1 secretion are induced in patients after AMI, especially in the group with impaired left ventricular function as determined by echocardiography. Also to assess whether these hormonal markers provide a more sensitive and specific index of left ventricular systolic function after AMI than clinical assessment.

3. investigate whether NT-proBNP and CT-1 may continue to be elevated post AMI in those patients with abnormal wall stress who may subsequently develop left ventricular dilatation. Thus, different patterns of CT-1 secretion may emerge, where secretion early in the course of AMI may be cytoprotective whereas continued secretion may result in ventricular dilatation and remodelling.

4. investigate whether plasma levels of NTproBNP and CT-1 are raised in patients with chronic HF and to correlate the levels of these peptides to the severity of HF. This would then look at the prospect of a non-radioactive test (immunoluminometric) test for NT proBNP being used as a screening test for LVD. We would also look at the influence of treatment (such as ACE inhibitors), renal impairment and valvular disease on these peptides.

These investigations may lead to :

1. The development of a rapid, sensitive, specific and cost-effective test for detection of LVSD in both the post-AMI patients and also patients with chronic HF enabling effective treatment to be instigated.

2. Further understanding of the role of CT-1 both in the acute phase following an AMI and its importance in the process of ventricular remodelling and chronic HF.

CHAPTER 5

MATERIALS AND METHODS FOR CT-1 ASSAY

We hypothesised that CT-1 is present in normal human plasma and increased in LVSD. We describe below the development of a competitive immunoluminometric assay for CT-1 and have used this assay to characterise a number of extracted plasma samples from patients with LVSD. Central to the widespread applicability of a biochemical test is the stability of the marker itself. The necessity for complex handling procedures in order to accurately measure levels of CT-1 would potentially limit its more extensive application as a research tool. In view of this we set out to assess the stability of CT-1 over a variety of time-scales and conditions.

PRINCIPLES OF IMMUNOCHEMILUMINESCENCE

All previous assays have made use of radioactivity for determining plasma concentrations of BNP, ANP or NT-proANP. An immnochemiluminumetric assay is similar in principle to radioimmunoassay whereby the label used is made to luminesce under favourable reaction conditions. Novel immunoluminometric assays were employed for detecting plasma NT-proBNP and CT-1 using a methyl acridinium ester as the chemiluminescent label.

The sensitive measurement of visible photons emitted during acridinium luminescence requires the use of a photomulitplier tube detector and is analogous to the measurement of radioactivity. The production of a proton is a result of a concerted intermediate reaction and acridinium chemiluminescence has been postulated to occur through a concerted

multiple bond cleavage mechanism involving a dioxetanone intermediate. The decomposition of this yields *N*-methylacridone in an excited singlet state which subsequently relaxes to its ground state with the emission of photons at a wavelength of approximately 430nm.

METHODS

Materials

An oligopeptide (referred to henceforth as CT) corresponding to amino acids 105-120 (CRRQAELNPRAPRLLR) of the human CT-1 sequence, representing the mid section of CT-1 was synthesised in the MRC Toxicology Unit, Leicester University. There is no homology between this oligopeptide and any other cytokine of the IL6 family. The peptide was further purified by high performance liquid chromatography (HPLC) on preparative C_{18} columns with an acetonitrile gradient, resulting in greater than 98% purity. The pure peptide was also confirmed to be of the correct predicted molecular mass using matrix assisted laser desorption mass spectrometry. Protein A Sepharose CL4B gel was obtained from Pharmacia, Hertfordshire. The biotinylating reagent sulfosuccinimidyl-6-(biotinamido)hexanoate was obtained from Calbiochem, Nottingham, U.K. The horse-radish peroxidase conjugated anti-rabbit IgG, the streptavidin-horse-radish peroxidase conjugate and the enhanced chemiluminescence (ECL) kits were obtained from Amersham International, UK. The methyl acridinium ester (4-(2-succinimidyloxycarbonyl ethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate) was a gift from Drs. Stuart Woodhead and Ian Weeks, Molecular Light Technology Ltd., Cardiff, U.K. The paramagnetic particles coated with goat anti-rabbit IgG was from Metachem Diagnostics Ltd., Northampton. The C_{18} plasma extraction

columns were obtained from Peninsula laboratories, Merseyside, U.K.[71,72,73]

Production of Antibodies

The CT peptide was conjugated to haemocyanin with the hetero-bifunctional cross linker ε-maleimidocaproic acid N-hydroxysuccinimde ester. Haemocyanin was dissolved in buffer (10 mg/ml in 100 mmol/l Na_2HPO_4 buffer, pH 8) and 1 ml was rapidly mixed with 400 nmoles of ε-maleimidocaproic acid N-hydroxysuccinimde ester (in 10 µl of dimethylformamide). After 30 min, the derivatised haemocyanin was gel-filtered on a Sephadex G25 column conditioned with 100 mmol/l Na_2HPO_4 buffer, pH 7.4. The haemocyanin was then mixed with 1 mg of CT-1 and the conjugation reaction proceeded for 3 h at room temperature. The conjugates were then dialysed with phosphate buffered saline (PBS) for 3 days at 4°C, with 4 changes of the PBS.

One rabbit was inoculated with subcutaneous injections of antigen (1 mg) emulsified with complete Freund's adjuvant. After a month, booster injections (0.5 mg) were given subcutaneously every 2 weeks and the antisera obtained after 3 months when titres had achieved good levels. The IgG fraction was obtained by protein A sepharose chromatography and is referred to as G187.

Peptide labelling with the methyl acridinium ester

The peptide CT was dissolved in 100 mmol/l Na_2HPO_4 buffer, pH 8 at a concentration of 200 µmol/l and 100 µl pipetted into an Eppendorf tube. Five µg of the methyl acridinium ester was dissolved in 5 µl of

dimethylformamide and mixed with 40 nmoles of the peptide. After incubation at room temperature for 30 min in the dark, 100 µl of a lysine quench solution (10 mg/ml in 100 mmol/l Na_2HPO_4 buffer, pH 8) was added and incubated for another 5 min. The labelled peptide solution was acidified with an equal volume of 1% trifluoroacetic acid (TFA). An aliquot of this was then injected onto a 3.9mm x 150mm Deltapak C_{18} 300 Angstrom column, mounted within a high performance liquid chromatography (HPLC) system (Waters, Watford, Hertfordshire) consisting of a Waters 600S controller, 626 pump and 486 tunable absorbance detector set at 215 nm to detect ultra-violet absorbance of the peptide peaks. The column was equilibrated with 0.1% TFA and a gradient of acetonitrile from 0 to 55% (at a rate of 2%/min) was used to elute the peptide. Unmodified CT was eluted at 37% acetonitrile. The methyl acridinium ester derivatised CT was eluted at 40% acetonitrile and the recovery of the labelled tracer amounted to mean ± SEM 40.8 ± 5.3% of the total label used. The hydrophobicity of the labelled peptide following derivatisation with the methyl acridinium ester facilitated the separation from unlabelled peptide. These fractions were collected and used for development of the immunoluminometric assays (ILMA). Fractions collected at other times (corresponding to $OD_{215\,nm}$ absorbance peaks) were not immunoreactive with the G187 antibody. The labelled peptides were stored in the dark at -70°C in the 0.1%TFA buffer, being stable for over 6 months. One preparation produced enough tracer for about 10,000 experiments.

Subjects and Blood samples

Blood was obtained from 16 normal controls and 22 patients with moderate to severe LVSD after informed consent. The studies were approved by the Leicester Health Authority Ethics Committee. Normal controls were out-

patients referred for echocardiography but had no significant cardiovascular abnormality. Wall motion index (WMI) was assessed using a nine segment echocardiographic model (see below) and used to assess left ventricular systolic function. All 16 normal controls (9 male, median age 40 years, range 20-79) had a WMI of 2 and no echocardiographic abnormality. None was receiving any treatment. Moderate to severe LVSD was defined as a WMI of less than or equal to 1.4 and the 22 patients (15 male, median age 68.5 years, range 43-83) with LVSD had WMI of 0.6 [0.2-1.4]. Five patients with LVSD were categorised within NYHA Class II, 13 patients within class III and 4 patients within class IV. Clinical LV failure (LVF) was present in 17 subjects. Sixteen patients were on loop diuretics, 12 were taking ACE inhibitors and 1 was on a β-blocker.

20 ml of blood was transferred to chilled tubes containing 500 IU/ml aprotinin (Trasylol, Bayer UK, Newbury) and EDTA (1.5 mg/ml). Following centrifugation, plasma was stored at -70°C until the assay was performed. All samples were analysed within 2 months of venesection.

Plasma specimens were defrosted and 1 ml was acidified with an equal volume of 1% TFA. After centrifugation, the supernatant was loaded onto C_{18} extraction columns. Following 2 washes (3 ml each) with 0.1% TFA, the peptides were eluted with 2 ml of 0.1% TFA containing 60% acetonitrile. The eluates were then dried in a centrifugal evaporator. The dried eluates were reconstituted in 1 ml of the ILMA assay buffer (see below) containing 0.1% Triton X-100 and assayed immediately.

12 patients (8 males, median age 64.5 years; range 21-84) were enrolled for involvement in the stability study. Seven patients had a confirmed diagnosis of myocardial infarction, three patients had a diagnosis of

crescendo angina and the other 2 had no cardiovascular history. This enabled studies on the stability of CT-1 over a broad range of values.

In the stability study 35 ml of peripheral venous blood was drawn from the ante-cubital fossa of each patient and collected into similar chilled polypropylene tubes containing EDTA and aprotinin and immediately placed on ice. The blood was divided into seven equal 5ml aliquots. One aliquot was centrifuged and frozen at -70°C immediately after collection. Other blood samples were stored for 24 and 48 hours at room temperature or on ice and then separated and stored at -70°C. Average room temperature measured at 1000hrs and 1700hrs on each day was 24°C.

Plasma extraction (see above) was performed prior to the assay and the samples after elution were evaporated to dryness under vacuum and stored at -70°C until assay. All samples were assayed (in duplicate) within 2 weeks of collection and within the same assay for all samples from each patient.

Immunoluminometric assay for CT-1

The ILMA assay buffer consisted of (in mmol/l) NaH_2PO_4 1.5, Na_2HPO_4 8, NaCl 140, EDTA 1 and (in g/l) bovine serum albumin 1, azide 0.1. Wash buffer was composed of (in mmol/l) NaH_2PO_4 1.5, Na_2HPO_4 8, NaCl 140, and (in g/l) Tween 20 0.5, gelatin 1, azide 0.1.

On day 1 of the assay, 100 µl of assay buffer containing 20 ng of the CT-1 antibody G187 was pipetted into tubes and incubated overnight at 4°C with 100 µl peptide standards in the range 1 - 2000 fmol per tube. All samples and standards were assayed in duplicate. 100 µl of assay buffer containing about 10^6 relative light units (RLU) of the labelled CT peptide

was then added and tubes again incubated overnight at 4°C. On day 3, 10 μl (10 μg) of paramagnetic particles coated with goat anti-rabbit IgG was added to tubes to recover the immunoprecipitates. The particles with attached immunoprecipitates were washed 3 times with the wash buffer described above (2 mls for each wash) and the particles recovered each time using a magnetised tube rack, allowing the wash solutions to drain adequately. Following the last wash, 100 μl of distilled water was added to the tubes and the particles resuspended by vortexing the tubes. Readings of the chemiluminescence from the immunoprecipitates were then obtained on a Lumino portable luminometer, (Stratec Electronic GMBH, Birkenfeld, Germany). In order to initiate chemiluminescence of the label [74,75], the first injection was 100 μl of 100 mmol/l HNO_3 containing 0.05% hydrogen peroxide, followed 4 sec later by an injection of 100 μl of 250 mmol/l NaOH containing 0.25% cetyl triethylammonium bromide. The detergent optimised the light emission from the label. Chemiluminescence was measured over 2 sec following the second injection and expressed as RLU. Standard curves were obtained and non-linear least squares fitting performed using an algorithm with a Rodbard 4 parameter equation.

Echocardiography

Echocardiography was performed on all subjects using a Hewlett Packard Sonos 1500 imaging system and recordings were on Panasonic Super VHS tapes. Left ventricular wall motion index (LVWMI, a regional measurement of LVD) which has been shown to be closely correlated to LVEF by radionuclide cardiography and invasive ventriculography [76,77] was calculated using a nine-segment model which was originally described by Heger et al [78]. The scale used for the LVWMI has been validated [79,80] and a linear correlation between LVWMI and LVEF has been previously demonstrated [80]. LVWMI multiplied by 0.3 gives an estimate of LVEF

[76]. This nine segment model for calculation of LVWMI was used in the large multicentre TRACE study.

The scanning protocol consisted of obtaining the following views :- (A) parasternal long axis sector of left ventricle, (B) parasternal short axis at mitral valve level, (C) parasternal short axis at papillary muscle level, (D) apical 4 chamber view, (E) apical long axis view. LVMMI was analysed blind to the patient details.

The severity of regional wall motion abnormalities was graded by a score for each segment:

- hyperkinesia = 3
- normokinesia = 2
- hypokinesia = 1
- akinesia = 0
- Dyskinesia (Paradoxical Motion) = -1

M-mode echocardiography was performed successfully on 23 subjects. Left ventricular (LV) chamber dimensions were obtained at the tip of the mitral valve. Interventricular septal thickness (IVST) and posterior wall thickness (PWT) were measured at end diastole. Left ventricular internal dimensions (LVID) were obtained at end diastole (LVIDd) and at end systole (LVIDs). Fractional shortening (FS) was calculated as the percentage change in the internal LV dimension between systole and diastole. LV volumes were calculated according to the formula of Teicholz et al [81]. LV stroke volume (SV) was the LV end diastolic volume (EDV) minus the LV end systolic volume (ESV).

Determination of molecular weight of CT-1 by protein blotting using sodium dodecyl sulfate-polyacrylamide gels

Since the epitope of CT-1 to which G187 was directed was similar in humans and rats, extracts from rat hearts were used as a positive control.

Rat hearts were homogenised in homogenization buffer consisting of (in mmol/L) EDTA 5, phenylmethyl sulphonyl fluoride 1, phenanthroline 1, iodoacetamide 1, (in μg/mL) pepstatin 1, leupeptin 2, in phosphate buffered saline with 0.1 % (v/v) Triton X-100. Following centrifugation at 600 g for 10 min at 4°C to remove nuclei, and then for 30 min at 10,000 g to remove mitochondria, the supernatant was recovered. This was acidified with an equal volume of 1% TFA and then loaded onto C_{18} columns and eluted as described below.

Plasma (10 ml from normal control and 5 ml from heart failure patients) was acidified with an equal volume of 1% TFA and following incubation on ice for 30 minutes, the samples were centrifuged. The supernatants were then loaded onto C_{18} columns (containing 1 g of the sorbent material). Following 5 washes with 4 ml each of 0.1% TFA, the peptides were eluted with 4 ml of 60% acetonitrile in 0.1% TFA. The samples were dried down in a centrifugal evaporator.

The dried samples (equivalent to 10 ml plasma from normal controls, 5 ml from heart failure patients and about 1/5 of a rat heart) were dissolved in 0.5 ml of 0.1 mol/l Na_2HPO_4 buffer (pH 7.4) containing 0.05% Tween 20 and 1 mmol/l EDTA. After sonication for 30 s, the samples were centrifuged at 3000 rpm for 10 min. The supernatants were then recovered into Eppendorf tubes and 20 μg of the CT-1 specific antibody G187 was added. The tubes were rotated end on end at 4°C for 6 h. 150 μl of the paramagnetic particles coated with goat anti-rabbit IgG (binding capacity >30 μg of rabbit IgG) was then added and rotation end on end continued at 4°C for 16 h. The immunoprecipitates of CT-1 were recovered using a magnetic rack and the paramagnetic particles were washed three times with PBS containing 0.1% Tween 20 (1 ml per wash).

The paramagnetic particles with attached immunoprecipitates were then treated with 100 μl of gel sample buffer (consisting of Tris 200 mmol/l pH 6.8, 20 g/l sodium dodecyl sulphate, 0.4 g/l coomassie blue, 40 % (by volume) glycerol, 10 mmol/l dithiothreitol) and boiled for 5 min. The particles were pelleted by centrifugation and the supernatants were then loaded onto 17% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. Coloured molecular weight markers were loaded to enable calibration of the observed bands. The proteins were then blotted over onto reinforced nitrocellulose (0.2 μm), and blocked overnight in 1% dried low fat milk powder in tris buffered saline (TBS, composed of Tris 20 mmol/l, NaCl 135 mmol/l) containing 0.1% Tween-20. In order to achieve detection of CT-1 without visualisation of the heavy chains of rabbit IgG, we prepared a biotinylated version of the CT-1 antibody G187.

Biotinylation of G187 was achieved by reacting 500 μg of G187 IgG (in 1 ml of 0.1 mol/l Na_2HPO_4 buffer (pH 7.4) containing 1 mmol/l EDTA) with 500 nmoles of sulfosuccinimidyl-6-(biotinamido)hexanoate for 4 h at room temperature. The reaction was quenched by addition of 100 μl of 10 mg/ml lysine (in PBS). The mixture was then ultrafiltered on 10 kD nanosep cut-off filters (Pall Filtron Corporation, Lichfield, Staffordshire, U.K.) with 10 changes of PBS buffer to separate biotinylated antibody from low molecular weight biotin products.

Detection of CT-1 on the blots was then achieved by incubating the nitrocellulose filters for 1 hour at room temperature with 2 μg/ml biotinylated G187 in TBS-Tween. After extensive washes with TBS-Tween, the secondary detection reagent (streptavidin-horse-radish peroxidase conjugate) was added at 1:1500 dilution in TBS-Tween and incubation was at room temperature for 1 h. Following further washes (10), the blot was developed using the Amersham enhanced chemiluminescence (ECL) kit as

described in the instruction manual. Blots were exposed onto pre-flashed X-ray films to visualise the immunoreactive bands.

Statistics

All statistical analyses were carried out using the software package Minitab (Minitab Inc, PA, USA). All results are expressed as medians [ranges]. Comparisons between LVD patients and controls were made using the Mann Whitney test. CT-1, patient age, ISVT, PWT, WMI, FS, ESV, EDV, SV and LVDId were normalized by log transformation. Pearson's correlation coefficients were also computed.

Differences among the various approaches for handling the samples before storage at -70°C were assessed by analysis of variance and the paired t test and corresponding 95% confidence intervals are reported. These confidence intervals were transformed into percentage differences to aid interpretation.

RESULTS

Following labelling of the peptide CT with the methyl acridinium ester, HPLC was essential to separate labelled from unreacted peptide. Peaks recovered at other acetonitrile concentrations on the gradient were unreactive with the antibodies. Although 10^6 RLU of label were added per tube, the non-specific binding was under 1000 RLU using the paramagnetic particles to recover immunoprecipitates and with the washing protocol described above. The quantity of antibody added to tubes was titrated so that a non-saturating amount was added to each tube. Figure 1 illustrates that RLU measured in immunoprecipitates recovered with differing amounts

of the antibody. On this basis, 20 ng was chosen as a suitable antibody amount which was not saturated for that particular tracer amount added.

Standard curves were constructed using known amounts of CT and 20 ng of the antibody. 10^6 RLU of label was subsequently added. Chemiluminescence of the recovered immunoprecipitates with differing amounts of added peptide standard is plotted in figure 2. Increasing concentrations of the peptide displaced the label competitively from the immunoprecipitates.

Further evaluation of the assay for CT-1 indicated no cross reactivity with other peptides (ie ANP, BNP, CNP or proBNP) and Interleukin-6 and LIF which are known to be elevated in heart failure (all under 0.1%). Peptide recovery using the C_{18} column plasma extraction process was 76.2 $\pm$ 3 %. Within assay coefficients of variation for different concentrations of CT-1 are reported in Table 1. Between assay coefficients are also reported in Table I, where the same preparations of CT-1 utilised for the within assay determinations were measured on 5 other occasions. The blank value of the assay (mean $\pm$ 2SD, using water) was 1.1 $\pm$ 0.8 fmol/ml.

Figure 2 illustrates a competitive binding curve for CT-1 together with 2 plasma extracts from patients with congestive cardiac failure, diluted with ILMA assay buffer. The dilution curves are parallel with the competitive binding curve for CT-1 within the working range of the assay suggesting that immunoreactivity of the CT peptide with G187 resembled that of natural CT-1.

Echocardiographic data obtained from LVD subjects and controls are reported in Table 2. Plasma CT-1 levels were elevated in patients with LVD

(median 86.0 [range 33.0-516.0] fmol/ml) compared to normal controls (median 28.7 [range 7.6-42.1] fmol/ml, P<0.0001, Figure 3).

Log CT-1 was related to the following categorical variables: clinical LVF (r=0.56, p<0.0001); current diuretic use (r=0.56, p=0.002); current ACE inhibitor use (r=0.42, p=0.008); and also to echocardiographic indices: log IVST (r=0.43, p=0.04); log PWT (r=0.47, p=0.02); LVIDs (r=0.55, p=0.007); log WMI (r=-0.75, p<0.001); log ESV (r=0.56, p=0.006); SV (r=-0.58, p=0.003); and log FS (r=-0.71, p<0.001) (Figure 4-10). Log WMI was correlated to log FS (r=0.83, p<0.001). There was no correlation with age or EDV with plasma CT-1 levels.

We considered log age, log CT-1, history of IHD or hypertension, current diuretic or ACE inhibitor use and presence of clinical LVF in a multivariate model of the predictors of log WMI. The only significant predictor was log CT-1 (p=0.001). On best subsets analysis the strongest correlates with log WMI were clinical LVF (R^2=56.6%) and log CT-1 (R^2=55.5%). Both clinical LVF and log CT-1 together improved diagnostic accuracy (R^2=71.6%).

We considered log age, history of IHD or hypertension, current diuretic or ACE inhibitor use, clinical LVF, log WMI, log IVST, log PWT, log ESV and log EDV in a multivariate model for the predictors of log CT-1. The only significant predictor was log WMI (p=0.007), with an R^2 of 55% on best subset analysis.

Results for the stability of CT-1 are presented in Table 3. There was a strong correlation between baseline log CT-1 level in plasma stored at -70°C immediately after sampling (baseline log CT-1) and log CT-1 at 24 and 48 hours at room temperature (r=0.85 & r=0.98; p<0.0001 for both). There was in addition a correlation between baseline log CT-1 levels and

log CT-1 at 24 and 48 hours when stored on ice ($r= 0.88$ & $r=0.94$; $p<0.0001$ for both). Our results demonstrate that CT-1 is stable in whole blood treated with EDTA and aprotinin for up to 48hrs at room temperature and when stored on ice.

The molecular weight of CT-1 was then determined by protein blotting on 17% SDS-PAGE gels. The biotinylated antibody G187 detected a protein of molecular weight of about 26.7 kD, which was more prevalent in plasma extracts from patients with LVD than controls (figure 11). In rat heart extracts, a similar major band of immunoreactivity was evident at 26.7 kD. A minor band of immunoreactivity was visible at 28 kD in both rat heart extracts and the human plasma samples. Similar results were obtained on 3 separate occasions.

DISCUSSION

We have devised a novel sensitive and specific non-radioactive competitive ILMA technique for CT-1, the advantage of a competitive assay being the ability to measure the total amount of CT-1 (both free and bound) in a process where plasma is extracted on C_{18} columns. The ILMA we have described has the advantage of using non-radioactive techniques that do not require extensive laboratory radiation safety measures. Most of the reported techniques using chemiluminescence have utilised labelled antibodies [75], and the current method is unusual in that a small peptide was successfully labelled for performing the competitive ligand binding assay, thus establishing the versatility of this labelling technique for antigens as well as antibodies. Moreover, the purified tracer is stable when stored at -70°C for over 6 months and is relatively inexpensive and easy to prepare. Coefficients of variation between and within assay are acceptable for a competitive assay.

This is the first quantitative assessment of plasma CT-1 in humans. The protein has been postulated to be important in cardiac development. Its presence in adult plasma is therefore a novel finding and its physiological role is currently unclear. Furthermore, the present study is the first demonstration of significant elevation of plasma CT-1 in patients with LVD. The molecular mass as estimated by SDS-PAGE is approximately 26.7 kD, consistent with the estimated molecular weight from its amino acid sequence. A minor band was also visible at 28 kD. Cytokines such as tumor necrosis factor-alpha, interleukin-1 alpha, interleukin-1 beta and interleukin-6 are capable of modulating cardiovascular function by a variety of mechanisms, such as promoting left ventricular remodelling [82,83]. However the exact role of CT-1 in LVD and its relationship to ventricular remodelling remains to be defined.

End stage heart failure due to ischaemic or dilated cardiomyopathy is characterised by a dilated, thin walled ventricle. The structural basis for this ventricular dilatation is the side-to-side slippage of myocytes However, probably of more importance is an increase in the myocyte length secondary to an increase in numbers of sarcomeres in series [84,85,86]. It has been seen that the degree of side-to-side slippage of myocytes is not correlated to the magnitude of ventricular enlargement and furthermore, the myocyte lengthening alone could account for all the ventricular dilatation seen in heart failure secondary to an ischaemic or dilated etiology [84,85,86]. Similar changes of myocyte lengthening and maladaptive remodelling of the cardiac myocyte shape begin long before ventricular failure occurs in hypertension [87,88].

It has been seen that CT-1 can activate a similar form of hypertrophy i.e. the promotion of sarcomere assembly in series via a gp-130 dependent

signalling pathway, as is seen in end stage cardiomyopathy of ischaemic, dilated or hypertensive origin. This secretion of CT-1 in heart failure may be deleterious, since the eccentric hypertrophy which results from addition of sarcomeres in series may lead to ventricular dilatation and irreversible loss of function (with similarities to the eccentric hypertrophy in volume overload situations). This therefore raises the strong possibility that CT-1 may play a central role in the process of ventricular dilatation and remodelling seen in heart failure.

The potential mechanism for increased circulating plasma CT-1 in patients with heart failure is unknown. One possibility is that CT-1 expression may be induced by myocyte stretch secondary to volume or pressure overload. Hypoxia may also be an additional factor capable of stimulating CT-1 release from myocytes.

This is also the first study to assess the stability of human CT-1. Our results demonstrate that CT-1 is stable in whole blood treated with EDTA and aprotinin for up to 48 h at both room temperature and when stored on ice. This stability of CT-1, confers the potential for its more widespread introduction into clinical investigation of heart failure.

Adult cardiac cells are terminally differentiated and therefore have lost their proliferative capacity. Long standing exposure to hypertension or haemodynamic stress secondary to ischaemic injury can activate a distinct form of myocardial hypertrophy resulting in the heart becoming dilated. At the myocyte level there is addition of new myocytes on series. This finally results in an irreversible loss of myocardial function. The identification of factors which mediate the onset of cardiac hypertrophy, remodelling and eventually end stage heart failure will always remain a major pursuit in cardiac molecular biology and medicine. This study has identified a new

signalling pathway which could help us to understand further the pathophysiology of heart failure and also may lead us to new therapeutic avenues in the treatment of heart failure.

The major limitation of this study is the inability to define a direct cause and effect relationship between the elevated CT-1 levels and the clinical parameters. It is also certainly possible that CT-1 levels may be elevated due to the ageing process alone. In our study the patients with heart failure were older than the control groups. However , there was no correlation between CT-1 and age in the study groups. Clearly additional studies with larger number of patients and a more detailed examination of echocardiographic parameters are required.

In summary, we have demonstrated the possibility of using a chemiluminescent methyl acridinium ester derivative to label a small cytokine (peptide) and developed a competitive immunoluminometric assay for CT-1 which is sensitive and specific. The robustness of this peptide under different storage conditions would enable further studies on the physiology and pathophysiology of this interesting cytokine to be performed. More extensive investigations on the role of CT-1 in cardiovascular disease are currently in progress using this novel immunoluminometric assay.

Table I Assay coefficients of variation for ILMA of CT-1

Within Assay Coefficients of Variation (n=5)

CT-1 (fmol)	Coefficient of Variation %
7.8	7.6
15.6	9.0
31.3	6.2
62.5	3.9
125	14.5

Between Assay Coefficients of Variation (n=5)

fmol CT-1	Coefficient of Variation %
1.9	8.0
3.9	9.6
7.8	6.2
15.6	13.1
31.3	10.3
62.5	7.4
125	10.9
250	11.4
2000	27.5

Table 2: Echocardiographic data for the 16 normal controls and 22 patients with moderate to severe LVD.

Echocardiographic data	LVD	Controls	P value
WMI	0.6(0.2-1.4)	All 2	‡
FS (%)	17.4(11.4-25)	44.85(30.2-59)	‡
IVST (cm)	1.19(0.91-2.04)	0.93(0.6-1.06)	†
PWT(cm)	1.13(0.91-2.04)	0.95(0.58-1.02)	†
LVIDd(cm)	5.01(3.44-7.41)	5.06(3.94-5.82)	NS
LVIDs(cm)	3.90(2.84-6.16)	2.88(1.98-3.35)	‡
Left atrium(cm)	4(1.92-8.37)	3.35(2.42-4.47)	*
ESV(ml)	65.9(30.6-191)	31.75(12.4-45.8)	‡
EDV(ml)	119(48.8-290)	121.5(67.5-157)	NS
SV(ml)	49.4(18.2-99)	95.85(48-138)	†

* p=0.05, † p<0.005, ‡ p<0.001

Table 3: Plasma concentrations of CT-1 at different storage conditions.

Storage conditions	Mean difference from baseline. fmol/ml(%)	95% CI fmol/ml	95% CI as % of baseline mean
24 hours at room temperature	0.22(0.51)	+9.62 to -9.17	+22.3 to -21.3
24 hours on ice	3.49(8.1)	+16.05 to -9.06	+37.3 to -21.0
48 hours at room temperature	-0.49(-1.14)	+1.81 to -2.79	+ 4.2 to -6.47
48 hours on ice	-2.09(-4.9)	+3.84 to -8.02	+ 8.9 to -18.6

Fig. 1. Relationship of the chemiluminescence of immunoprecipitates using different concentrations of the CT-1 antibody in the immunoluminometric assay. Approximately 10^6 RLU of the labelled peptide CT were added per tube at the beginning of the assay.

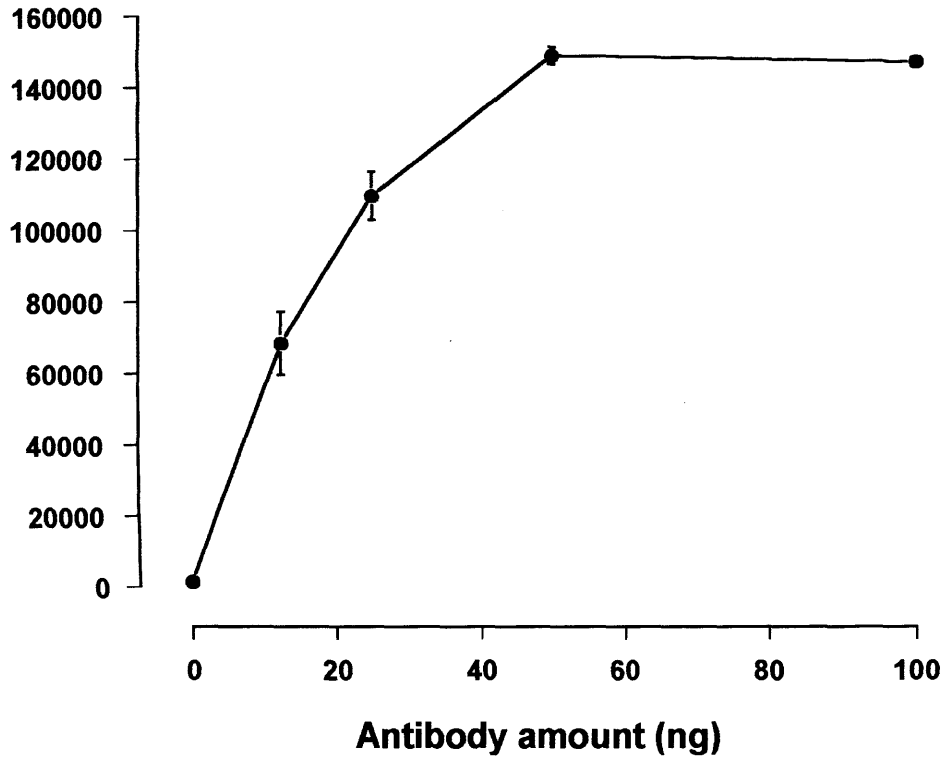


Fig. 2. Competitive binding curves for different added amounts of the peptide CT reacted with 20 ng per tube of the specific antibodies. The chemiluminescence values were corrected to a value of 1 in the tubes with no added peptide (to produce the ratio B/B_0). In addition Examples of 2 plasma samples from cardiac failure patients taken through a series of dilutions are also plotted as solid triangles or hollow circles with dotted lines.

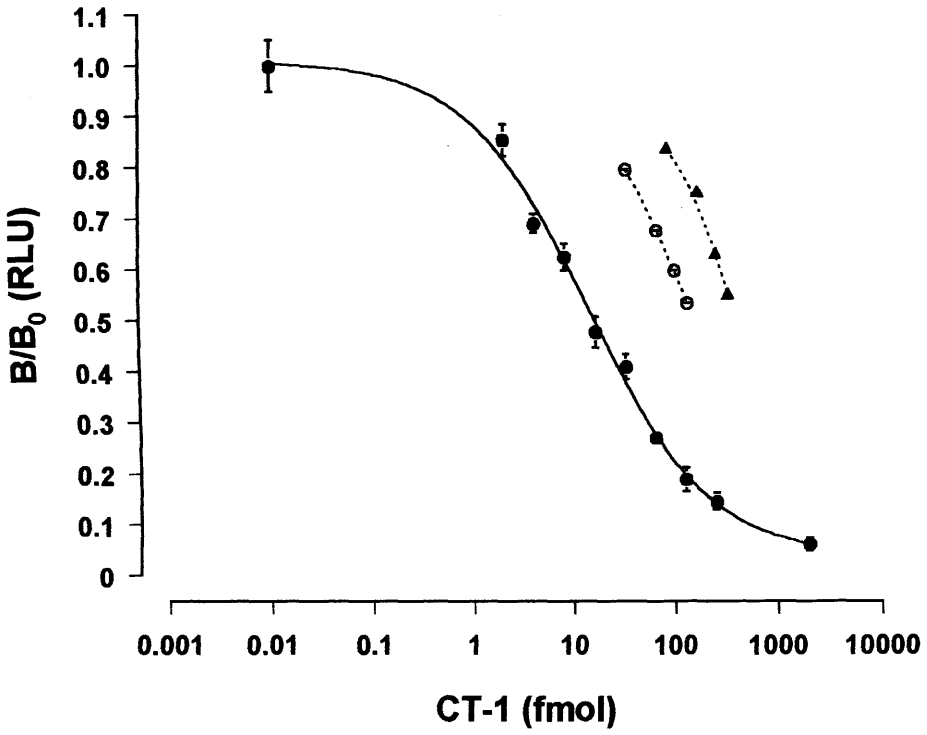


Fig. 3. Plasma levels of CT-1 in control subjects and patients with LVD. The medians are shown in solid bars and the values are significantly different ($P<0.001$).

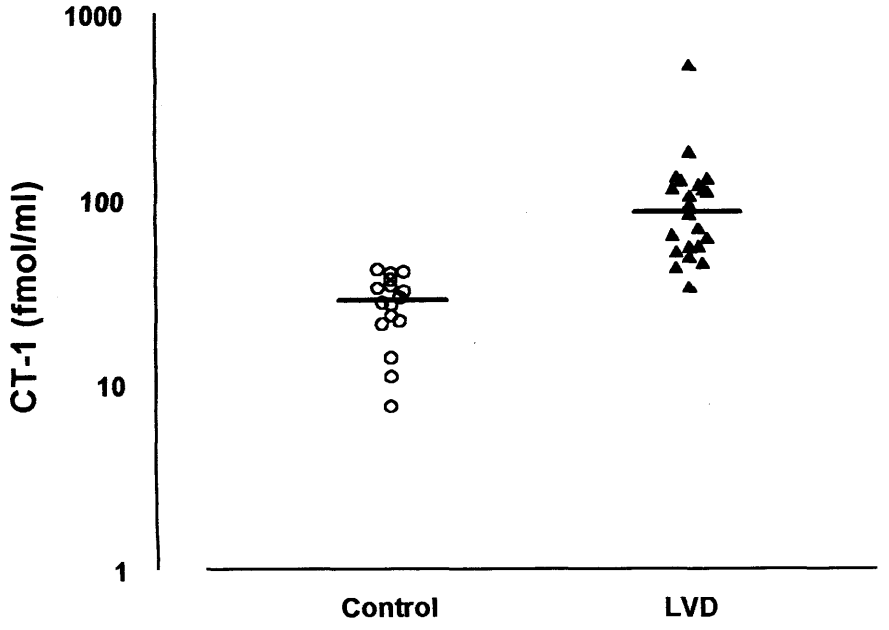


Fig. 4. Correlation between log CT-1 and log IVST.

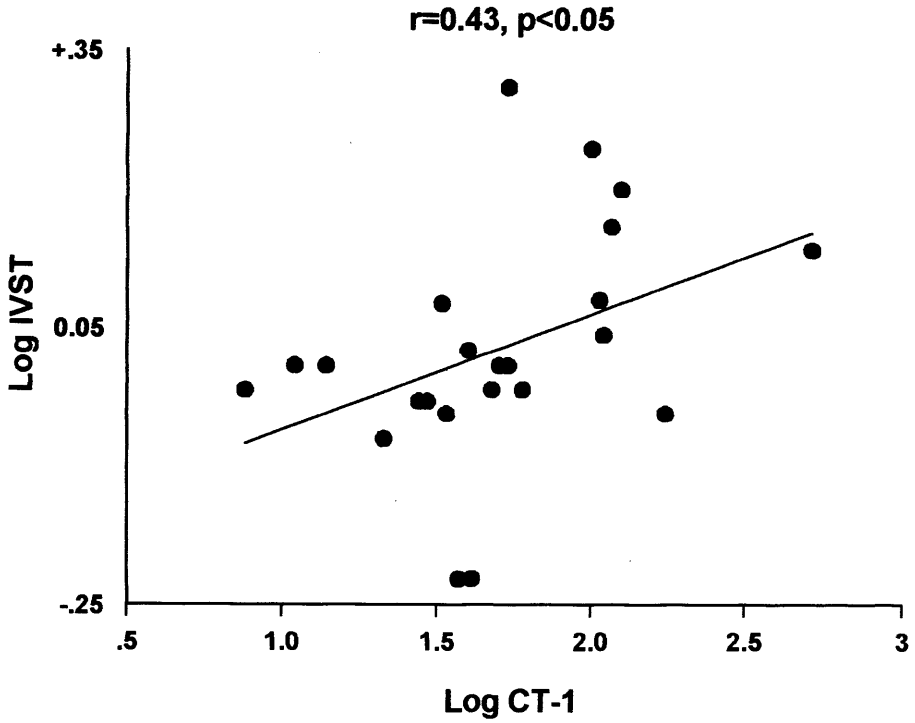


Fig. 5. Correlation between log CT-1 and log FS.

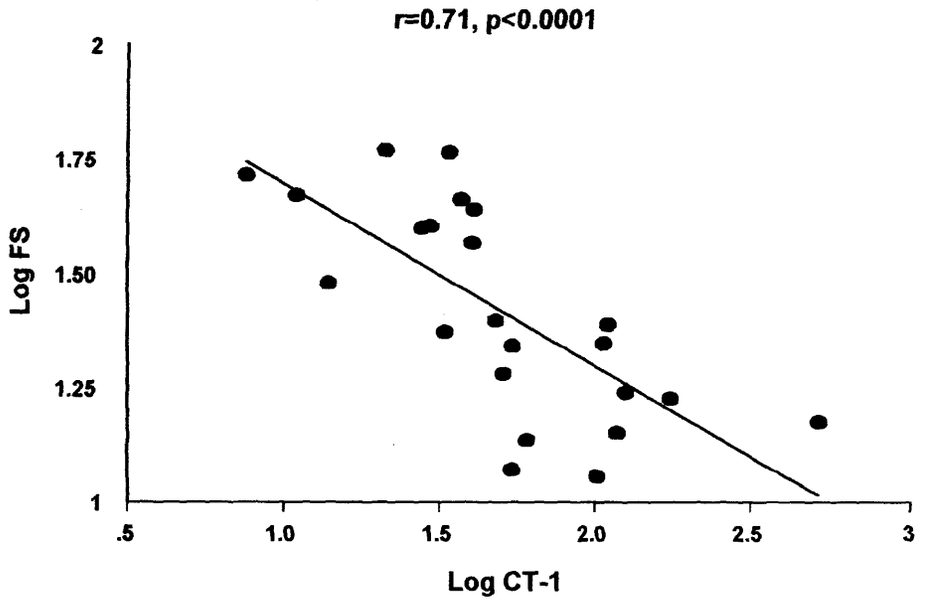


Fig. 6. Correlation between log CT-1 and log ESV.

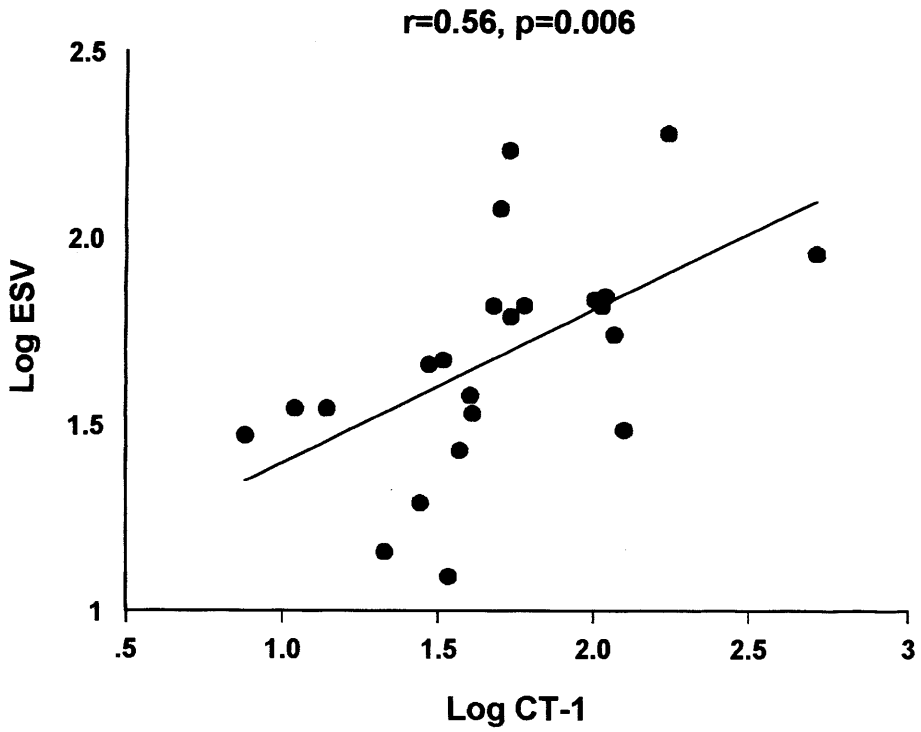


Fig. 7. Correlation between log CT-1 and log WMI.

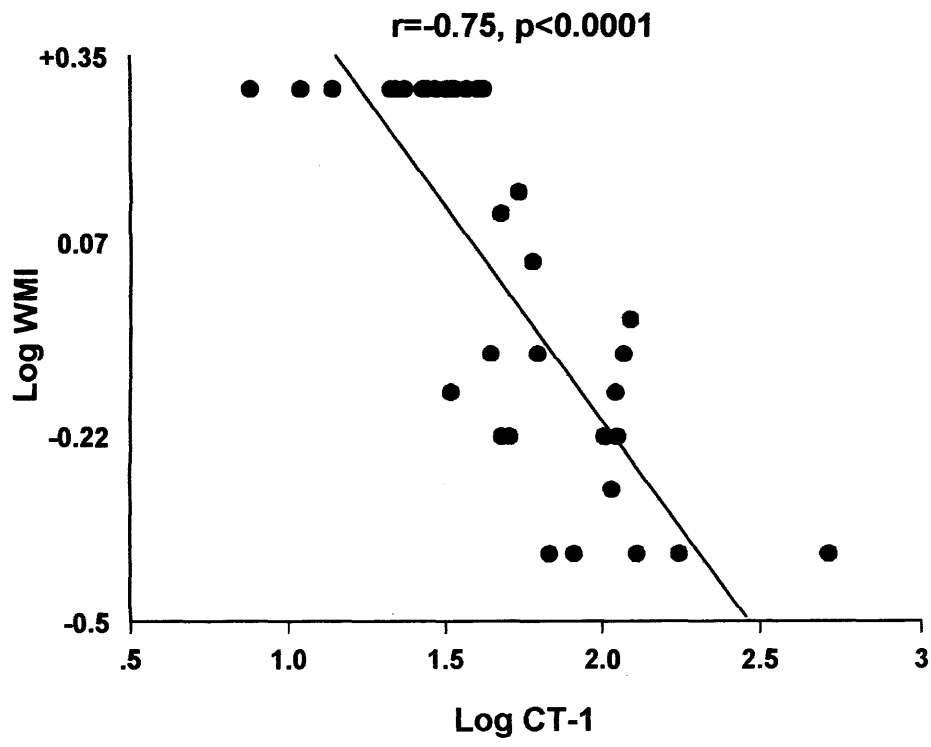


Fig. 8. Box plot showing Log CT-1 levels in those subjects with a history of IHD vs. Those who gave no history of IHD.

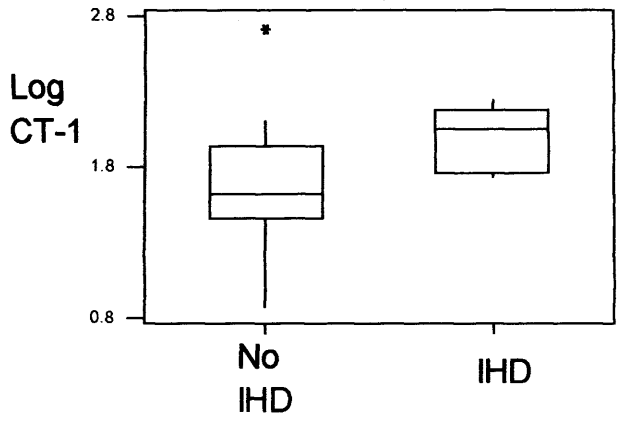


Fig. 9. Box plot showing Log CT-1 levels in those subjects with a history of HT vs. those who gave no history of HT.

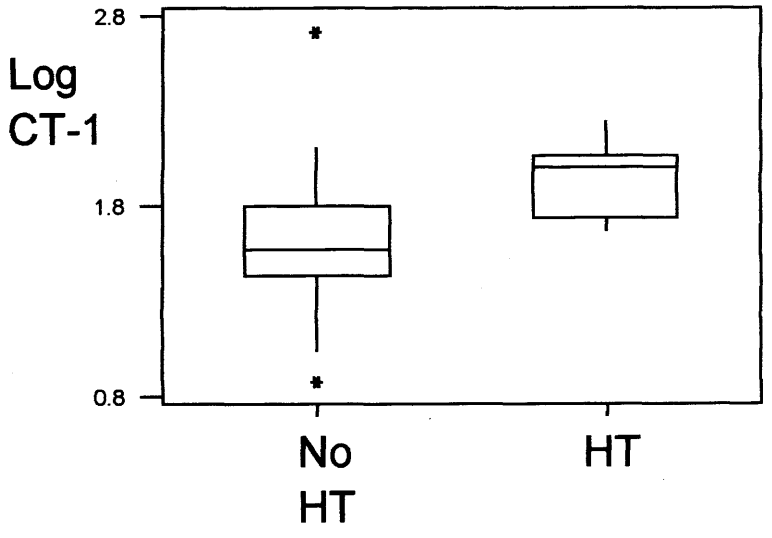


Fig. 10. Box plot showing Log CT-1 levels in those subjects with clinical LVF vs. Those without clinical LVF.

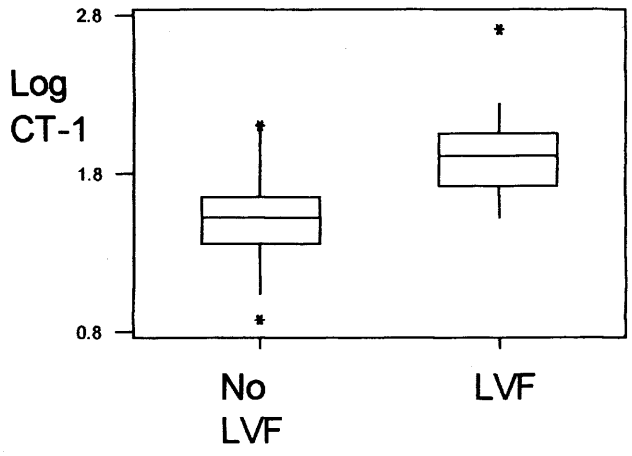


Fig. 11. A 17% -SDS-PAGE gel with 2 immunoprecipitates from plasma extracts (5 ml from one patient with LVD and 10 ml from one normal subject CON) resolved and blotted onto nitrocellulose was probed with the CT-1 antibody. Extract purified from rat heart is denoted RH. Coloured protein molecular weight standards are marked. One major band of immunoreactivity at 26.7 kD and a minor band at 28 kD are visible.

*** For figure see next page**

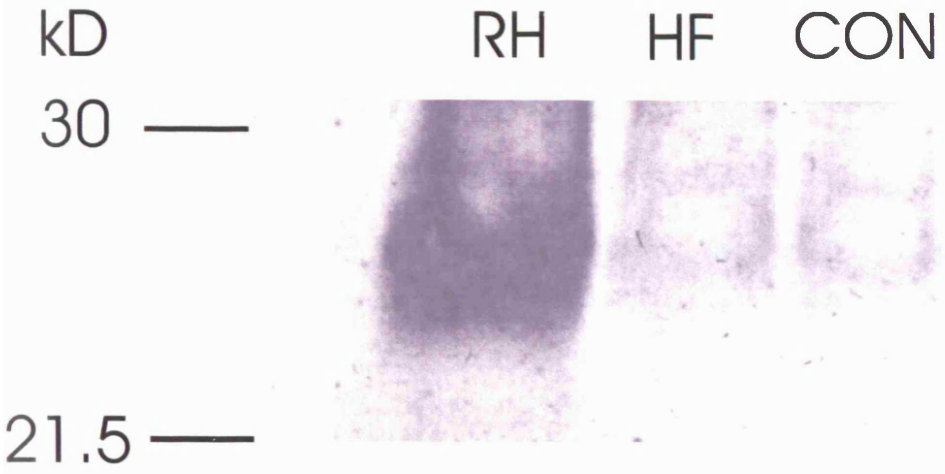

kD
RH
HF
CON
30
21.5

Figure 12: Correlation between log CT-1 concentration in plasma stored immediately at -70°C or kept for 48 hours as whole blood at room temperature. ($r = 0.89$, $p<0.0001$)

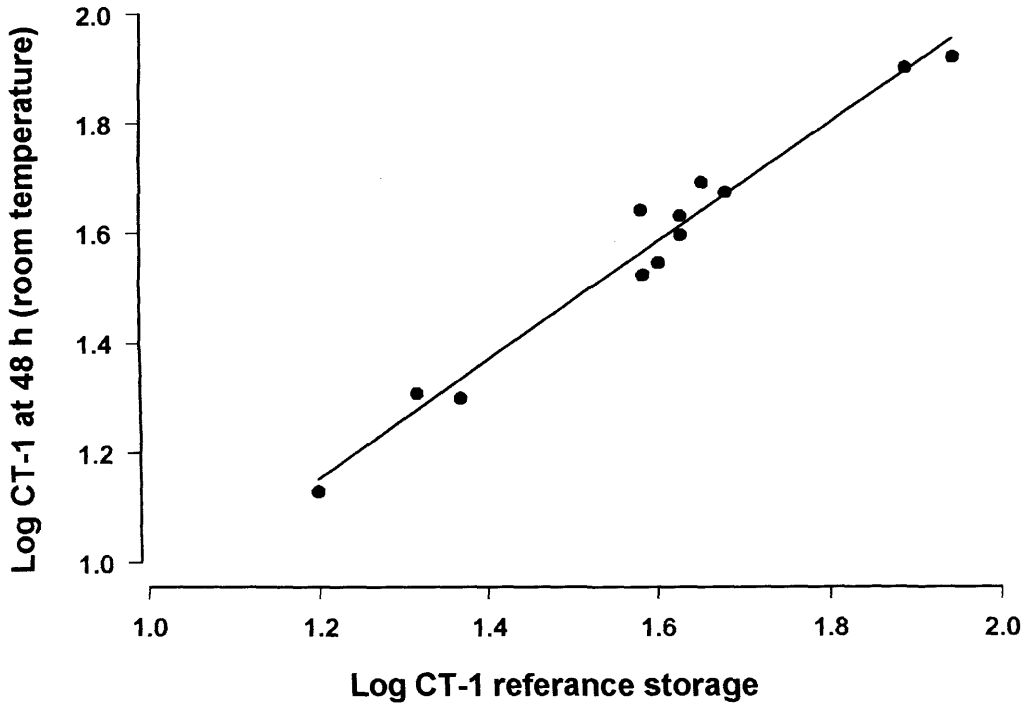
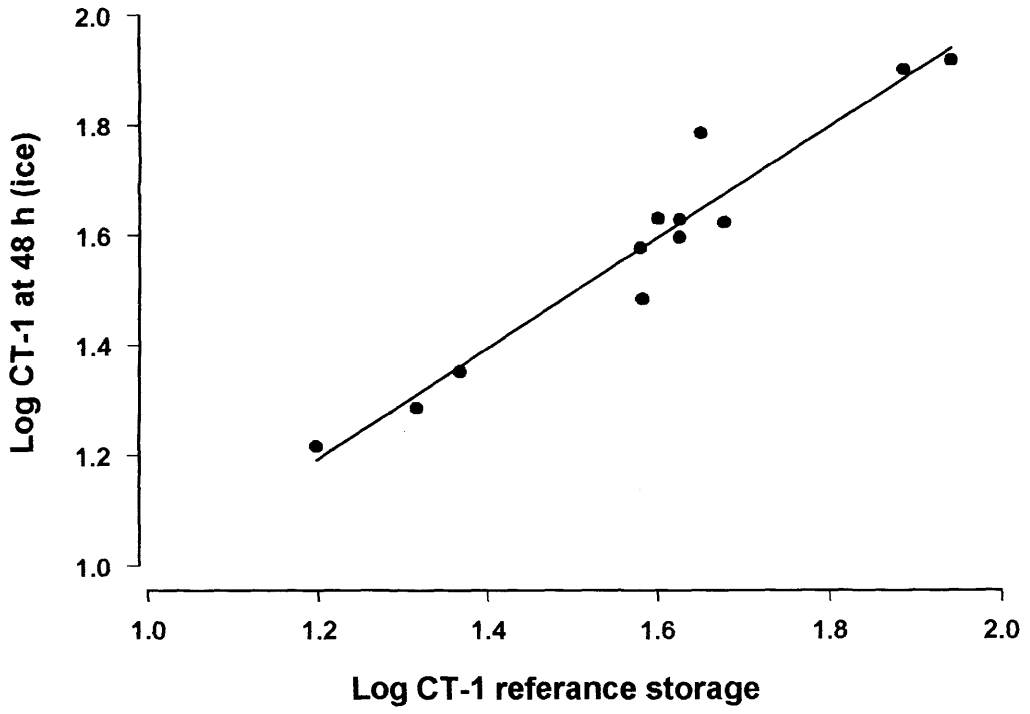


Figure 13: Correlation between log CT-1 concentration in plasma stored immediately at -70°C or kept for 48 hours as whole blood on ice. ($r = 0.94$, $p<0.0001$)



CHAPTER 6

MATERIALS AND METHODS FOR NT proBNP ASSAY

Our methodology for the assay of NT-proBNP has been described previously (89). We used an in-house rabbit, anti-human NTproBNP polyclonal antibody directed against a domain in the C-terminal section of NTproBNP (amino acids 65-76). The peptide was labeled using the chemiluminescent label 4-(2-succinimidyl oxycarbonylethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate and assayed on a LB953 luminometer (Berthold, Germany) (19). NTproBNP levels were determined blind to patient details. Each NTproBNP value represents the mean of duplicate measurements. The assay has been demonstrated to be specific for NTproBNP and unreactive with ANP, BNP or CNP (89). The within assay and between assay coefficients of variation are shown in table 1.

The concentrations of NT proBNP recorded in our study, utilising an antibody against the C- terminal of NT proBNP are considerably higher than the values determined in a previous study in which the antibody was directed against the N terminal of NT proBNP [53]. Recently the N-terminal domains of preproBNP have been demonstrated to oligomerise through leucine 'zipper-like coiled-coil' motifs [90] (a short bundle of peptide α-helices that are wound into a superhelix) which function to promote the formation of peptide oligomers. It is possible that an antibody directed against N-terminal domains of NT proBNP is potentially hindered from binding to its equivalent amino acid sequence. C-terminal domains however may be more readily accessible and detectable by immunoassay. Such differences in the differential immunoreactivities of the N- and C-

terminals of NT proBNP are likely to account for the disparity between the concentrations of NT proBNP observed between the two studies.

The antibody used in our work is specific for NT proBNP, as demonstrated by the immunoreactivity of the antibody on SDS-Page Gels and the insignificant cross reactivity of the assay with ANP, BNP-32 and γ-BNP [89]. Additionally the chemiluminescent assay is a simple and inexpensive to perform and does not require the extensive safety measures of conventional radioimmunoassay. This furnishes the ease of clinical application of this assay.

Furthermore we have shown that NTproBNP is stable in whole blood for upto 48 hours both at room temperature and on ice [91].

Table 1 Assay coefficients of variation for ILMA of NTproBNP

Within Assay Coefficients of Variation

fmol NT proBNP	Coefficient of Variation %
12.5	3.4
25	3.0
50	3.6
100	6.6
200	6.0

Between Assay Coefficients of Variation

fmol NT proBNP	Coefficient of Variation %
3.3	5.8
10	5.3
30	11.2
90	8.9
270	20.2
810	18.5
2430	18.8

Fig. 1. Competitive binding curve for the peptide NTproBNP, with 20 ng of the antibody G185 added per tube. Examples of 2 plasma samples from cardiac failure patients and 1 from a normal control taken through a series of dilutions are also plotted as hollow symbols with dotted lines.

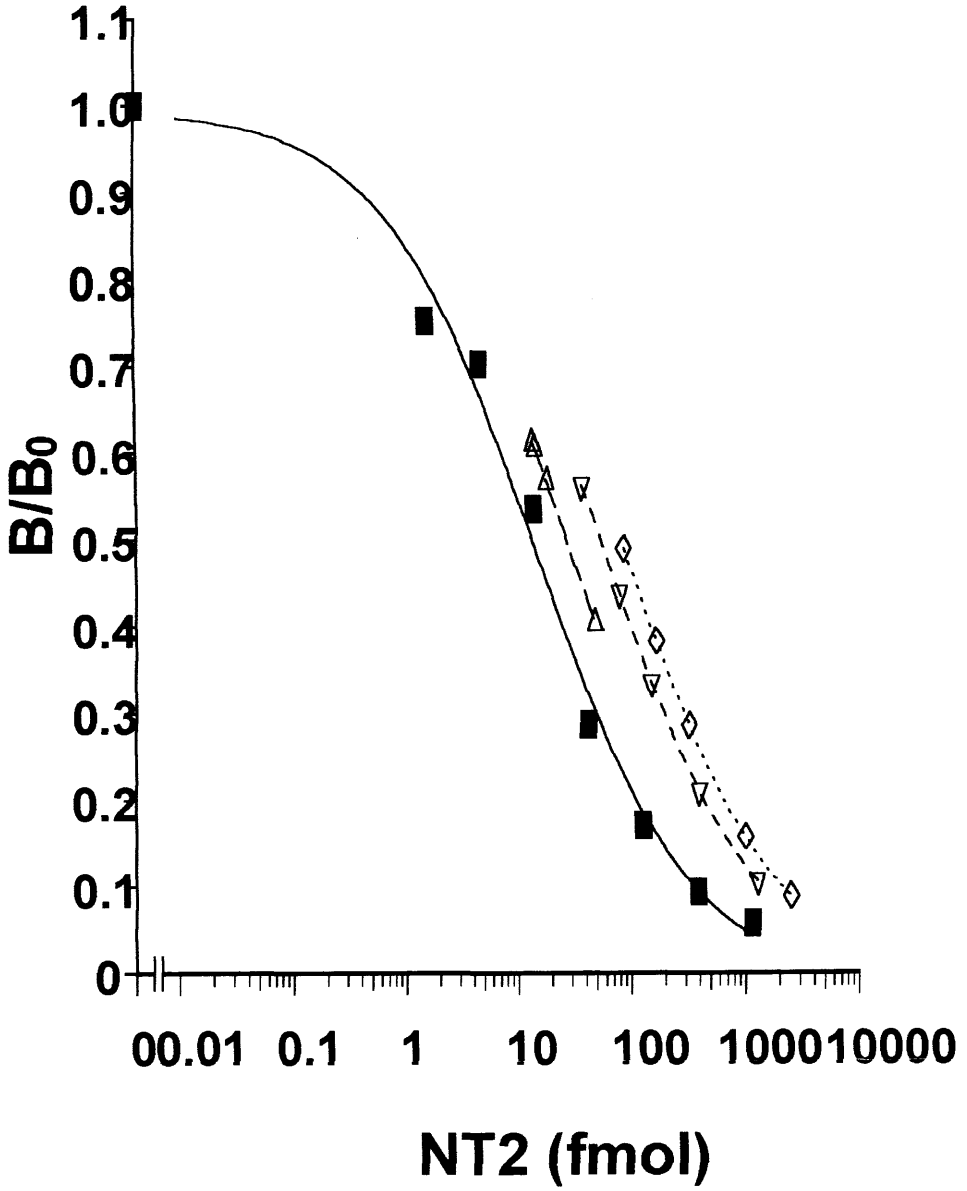


Figure 2: Correlation between log NT-proBNP concentration in plasma stored immediately at -70°C or kept for 48 hours as whole blood at room temperature.

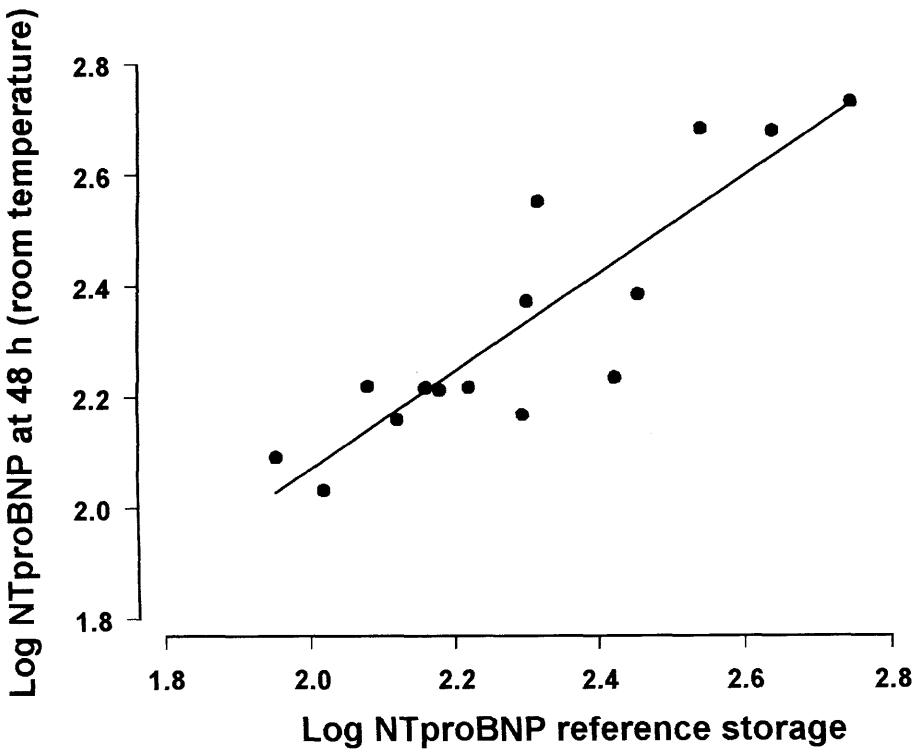
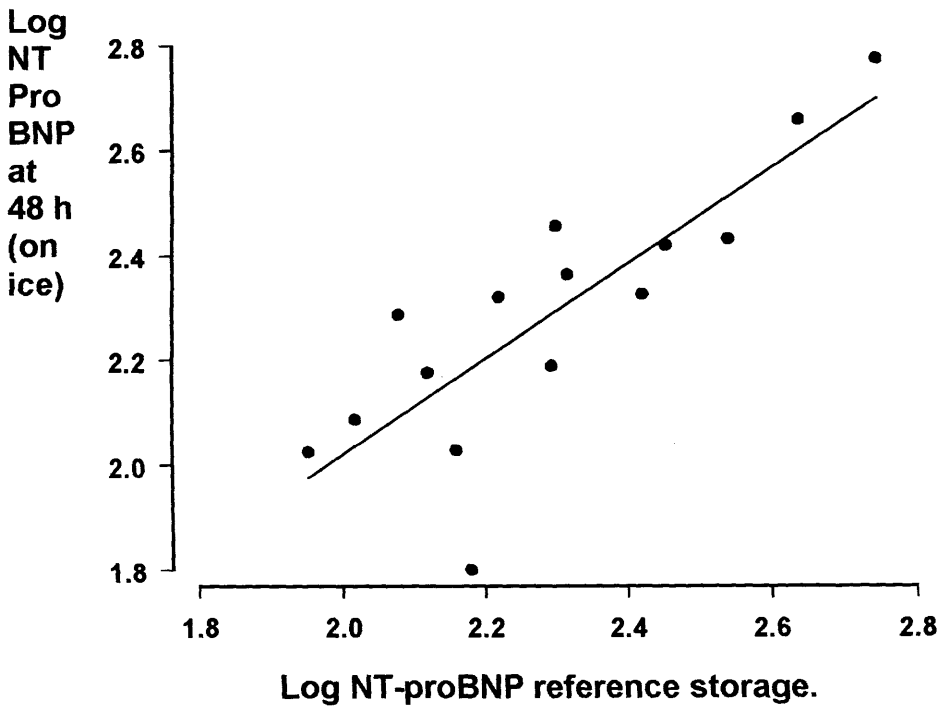


Figure 3: Correlation between log NT-proBNP concentration in plasma stored immediately at -70°C or kept for 48 hours as whole blood on ice.



CHAPTER 7

Plasma NT Pro-BNP and the ECG in the assessment of LVSD in a high risk population

INTRODUCTION

Plasma levels of BNP-32 may be superior to NT-ANP in screening for LVSD in the general population [47], in patients with chronic HF [92] and following AMI [53]. The N-terminal 76 amino acids of prepro-BNP (NT-proBNP) circulates in plasma in higher concentrations than BNP-32 in patients with HF [51] and in asymptomatic LVSD [52]. Plasma NT-proBNP measured 2-4 days following AMI predicts LV function and 2-year survival [53]. Thus NT-proBNP may be more useful than BNP-32 as an indicator of LVSD. However, some studies have failed to show correlation between parameters of LV function and plasma BNP levels [93]. Others have been criticized on the grounds of failing to account for confounding variables which may influence BNP levels such as drug therapy and renal impairment [47]. Moreover it has been suggested that in survivors of AMI, plasma BNP cannot discriminate between mild or moderate impairment of LV function on the one hand and preserved function on the other [94]. It has also been suggested that an abnormal ECG may identify patients with suspected LVSD in whom echocardiography is indicated. A number of studies have suggested that an abnormal ECG virtually excludes LVSD [31, 95]. Other studies have suggested that the ECG may be normal in over 10% of patients with significant LVSD [32].

The main aim of the current study was to test the diagnostic usefulness of circulating levels of NT-proBNP and the ECG as indicators of LVSD in an appropriate population, i.e. a cohort of patients referred by primary and secondary care physicians for echocardiography. In addition we examined the relationship between left ventricular systolic function and a number of routinely available clinical and laboratory parameters including those known to influence circulating natriuretic peptides levels in an attempt to define clinically useful predictors of impaired LV systolic function. We assayed NT-proBNP using a sensitive, immuno-luminometric method [89].

METHOD

Subjects

We studied 249 consecutive subjects referred for echocardiography to the Cardiology Services department at Leicester Royal Infirmary. Of these, 243 subjects yielded analysable echocardiograms (129 male, median age 73 years, range 20-94). The majority of the subjects (195) were hospital in-patients at the time of the scan. Patients were selected for study if there was (i) clinical suspicion of heart failure (n=125); (ii) history of ischaemic heart disease i.e. history of myocardial infarction, presence of pathological Q wave on the ECG, physician diagnosed angina or current use of an oral or sublingual nitrate (n=85); (iii) history of hypertension (n=101); (iv) history of shortness of breath in the absence of chronic airways disease (n=132); (v) current diuretic (n=117), angiotensin converting enzyme inhibitor (n=63) or digoxin (n=29) use. Patients were excluded if there was a history of recent (within 1 month) acute myocardial infarction.

The study was approved by the local ethical and research committee and all subjects gave informed consent.

Echocardiography

Echocardiography was performed by a single operator (ST) using a Hewlett Packard Sonos 1500 imaging system. Left ventricular wall motion index (LVWMI), was calculated using a nine-segment model [78]. As in previous large studies [6] we defined LVSD as LVWMI = 1.2. LVWMI was analysed by a single investigator (ST) blind to patient details and NTpro-BNP results.

Colour flow Doppler recordings on the parasternal, apical 4 chamber and the apical long axis views enabled semi-quantitative assessment of the severity of mitral, tricuspid and aortic regurgitation.. Mitral regurgitation (MR) was graded as absent=0, trace = 1, moderate = 2 or severe = 3 on color flow Doppler analysis based on the area of the jet projecting into the left atrium [96].

Immunoluminometric assay (ILMA) for NT-proBNP

The methodology for assay of NT-proBNP has been described previously (89). The normal range for NTproBNP in our laboratory is < 200 fmol/ml.

Electrocardiogram (ECG)

Only ECGs performed within 2 weeks of the echocardiogram were analysed and were available for 222 / 243 (91.3%) of the subjects. Each ECG was categorised as normal, minor abnormality (sinus bradycardia, sinus tachycardia, poor R wave progression, right axis deviation, non-specific ST/T changes, 1st degree heart block, or atrial enlargement) or major abnormality (atrial fibrillation, evidence of a past myocardial

infarction, voltage criteria for left ventricular hypertrophy, left axis deviation or left bundle branch block) [31].

Relationship between LVWMI and physiological variables

Concentrations of NTproBNP, serum creatinine and LVWMI were not normally distributed and were log transformed before analysis. For the categorical variables gender (male/female), past history of ischaemic heart disease (IHD/no IHD) or hypertension (HT/no HT), current use of diuretic (Diuretic /no Diuretic), ACE inhibitor (ACEI/no ACEI), β-blocker (βB/ no βB) and digoxin (Dig/no Dig), ECG (normal/minor abnormality /major abnormality) and severity of mitral regurgitation (none/mild/moderate/severe), log-LVWMI were compared and 95% confidence intervals calculated for the difference in medians between groups for each variable. The strength of association between LVWMI and each of the continuous variables log NTproBNP, age, aortic valve gradient and log creatinine concentration was quantified using the Pearson rank correlation coefficient. Predictive models for the response variable (LVWMI) were developed using multiple linear regression analysis and stepwise logistic regression analysis. All statistical analyses were carried out using the software package Minitab (Minitab Inc., PA, USA). Comparisons with $p<0.05$ were considered significant.

Receiver operating characteristic (ROC) curves were constructed to assess the ability of NT-proBNP throughout the range of concentrations to detect LVSD. The area under the curve, estimated by the method of Hanley and McNeill [97], provides a measure of the overall diagnostic accuracy of the test.

RESULTS

The characteristics of the study population (243 subjects) are shown in Table 1. 96 (39.5%) of the patients had LVWMI $\leq$ 1.2. Of these, 64 (66.6%) were taking a diuretic, 43 (44.7%) an ACE inhibitor, 76 (79.1%) gave a history of shortness of breath and 71 (73.9%) had clinical evidence of heart failure. Concentration of NTproBNP (median [range]) was higher in subjects with LVWMI $\leq$ 1.2 (509.6 fmol/ml [195.2-1619.3]) compared to those with of LVWMI $\geq$ 1.3 (255.2 fmol/ml [65.5- 1175.9] fmol/ml; $p<0.0001$).

Univariate analysis

On univariate analysis for the whole study population there was a negative correlation between log NTproBNP and LVWMI ($r=-0.624$, $p<0.001$) (Figure 1). LVWMI also correlated with age ($r= -0.15$, $p=0.01$, figure 4), serum creatinine ($r=-0.29$, $p<0.001$, figure 3), past history of IHD ($r=0.29$, $p<0.001$, figure 9), male gender ($r=0.23$, $p<0.001$, figure 5) and current treatment with diuretic ($r=0.34$, $p<0.001$, figure 6), or ACE inhibitor ($r=0.38$, $p<0.001$, figure 7).

132 (54%) patients were treated with ACEI and/or diuretic (48 both, 69 diuretic alone, 15 ACEI alone). Those treated were older (74$\pm$10 yrs) than those untreated (66$\pm$17 yrs, $p< 0.0005$). NTproBNP levels (fmol/ml; Median, Range) were similar in untreated (368, 78-1047) compared to patients receiving either ACEI or diuretic (355, 65-1619), ACEI alone (382, 84-1619), diuretic alone (398, 65-1609) or both (332, 124-1293)($p=0.981$, Kruskal-Wallis). LVWMI did not differ among groups ($p=0.941$, Kruskal-Wallis). The correlation between log NTproBNP and LVWMI was similar for

both treated (r=-0.661,p< 0.005) and untreated (r=-0.584, p<0.005) patients (Figure 2).

Multivariate analysis

On multiple regression analysis in the whole population, log NTproBNP (p<0.001), age (p=0.015), diuretic use (p=0.002), ACE inhibitor use (p=0.001) and male gender (p=0.026) were independently associated with low LVWMI. Serum creatinine (p=0.09) and abnormal ECG (p=0.27) were not predictors of LVWMI in the multivariate model. Using best subsets analysis, log NTproBNP alone (R^2=39%) was a better predictor of LVWMI than any other single factor (ACE Inhibitor R^2= 15%; mitral regurgitation R^2 = 15%; diuretic R^2= 12%) factor. The predictive value of the model was improved slightly by consideration of combinations of variables, all of which included log NTproBNP: log NTproBNP + ACE inhibitor R^2=44%; log NTproBNP + ACE inhibitor + male gender R^2=47%; log NTproBNP + ACE inhibitor + male gender + diuretic R^2=48%.

Independent predictors of LVWMI in patients treated with ACEI and/or diuretic were NTproBNP (p<0.001) and age (p<0.001). Using best subsets analysis, log NTproBNP alone (R^2=26.3%) was again the best predictor of LVWMI than any other single or combination of factors. In untreated patients only NTproBNP (p< 0.001) independently predicted WMI. On best subsets analysis, log NTproBNP alone (R^2=42.8%) was the best predictor of LVWMI. Thus, in both treated and untreated patients, log NTproBNP accounted for a substantial proportion of the total variance in LVWMI. There was a weak but statistically significant correlation between plasma NTproBNP and log creatinine concentration (r=0.28, p<0.001).

Figure 11 shows the ROC curve for various concentrations of NTproBNP in the diagnosis of LVSD in the whole study population. Plasma NTproBNP >275 fmol/ml predicted LVWMI=1.2 with a sensitivity of 93.8%, specificity of 55%, positive predictive value of 58% and a negative predictive value of 93%. The area under the ROC curve in the whole population was 0.854. A similar area under the curve of 0.856 was seen for NT-proBNP in the diagnosis of LVSD and/or moderate to severe mitral regurgitation.

Etiology of LVSD

In the study 47 patients were identified with a history of IHD and no history of HT and 63 had a history of HT with no history of IHD. LVWMI was lower in the IHD group compared to the HT group (Median 1, range 0.2-2 vs. Median 1.8, range 0.2-2, $p<0.005$). NT-proBNP was higher in the IHD group as compared to the HT group (Median 446.4, range 121.1-1292.6 vs. Median 336.5, range 79.2-1052.8, $p<0.005$).

The ECG in screening for LVSD

In this largely hospital based population, a normal ECG was found in 36/222 (16%), a minor abnormality in 36/222 (16%) and a major abnormality in 150/222 (68%). Of the 36 patients with normal ECG, 18 had LVWMI of 2, 6 (17%) had LVWMI of 1.3-1.9 and 12 (33%) LVWMI of = 1.2. Thus the ECG alone would have failed to identify 18 patients (50%) in whom LV function was impaired. In all of these patients NTproBNP was > 275 fmol/ml. Of the further 36 patients with minor ECG abnormalities, 10 (28%) had LVWMI of 1.3-1.9. NTproBNP was > 275 fmol/ml in 7/10. 15/36 (42%) of patients had LVWMI of = 1.2 and of these NTproBNP was > 275 fmol/ml in 13. Of the 150 patients whose ECG showed a major abnormality 52 had LVWMI = 2, in 29 of whom NTproBNP was < 275 fmol/ml.

Influence of mitral regurgitation

MR was graded as 0 in 44 (18%), 1 in 145 (60%), 2 in 40 (16%) and 3 in 14 (6%) patients. The estimated severity of MR correlated with plasma NTproBNP ($r=0.389$, $p<0.0005$). Mean NTproBNP differed among groups ($p<0.0005$, Kruskal-Wallis) and between all pairs except MR=2 and MR=3. (Figure 10). However there was a correlation between the severity of MR and WMI ($r=-0.365$, $p<0.0005$). Thus although elevated NT proBNP largely reflects reduced WMI it is affected by the presence and severity of MR.

DISCUSSION

Our study has confirmed the previously established relationship between plasma levels of natriuretic peptides and measures of left ventricular dysfunction [45,46,47,51,52,53,92]. Moreover our study indicates that plasma NTproBNP remains a strong predictor of left ventricular function when confounding variables such as renal function and concomitant drug therapy are taken into account. Our study also indicates that, at least in a population at relatively high risk of left ventricular dysfunction, consideration of the ECG does not add to the predictive value of plasma NTproBNP.

Our study has confirmed a linear relationship between NTproBNP and left ventricular function as assessed by LVWMI, a relationship previously reported for LV ejection fraction [53]. We have demonstrated the strong, independent predictive value of plasma NTproBNP in the identification of LV dysfunction. Previous studies on the use of natriuretic peptides for

detecting LVSD have utilized echocardiographic ejection fraction and fractional shortening [45,46] or radionuclide ventriculography [53]. Echocardiographic measures of LV dysfunction have with rare exceptions failed to predict prognosis in multivariate models [98]. This perhaps reflects the inaccuracy of current methods for measuring ejection fraction. On the other hand, the LVWMI is a relatively simple, robust and reproducible measurement [80] and the nine segment model has been shown to contain prognostic information in patients with HF [99].

While the study of Hunt et al [52] indicated a correlation between NTproBNP and creatinine clearance, none of the other studies of the natriuretic peptides in the identification of LVSD have addressed the influence of renal function and concomitant drug therapy on plasma hormone levels. Our study has addressed both of these issues. We identified a correlation between log creatinine and log NTproBNP. However we also identified a correlation between log creatinine and LVWMI ($r=-0.29$, $p<0.001$). Moreover, while creatinine concentration was a univariate predictor of LVWMI, this parameter was not significant on multivariate analysis. While we accept that serum creatinine concentration represents a crude measure of renal function, this is the measure that is usually applied clinically. Moreover we have no reason to think that a more sensitive measure of renal function such as creatinine clearance would have significantly reduced the predictive value of NTproBNP in our population.

Concerns have been expressed that drug treatment, in particular diuretic or ACE inhibitor therapy [100], β-blockers [94, 101], and digoxin [102] may modify plasma levels of natriuretic peptides and nullify their potential as markers for LVSD. We have observed the correlation of NTproBNP with LVWMI to be as strong in the group who were being treated with a diuretic or an ACEI when compared to those patients who were receiving neither.

Similarly we detected no influence of concomitant β-blocker or digoxin therapy. Although our study does not indicate whether NTproBNP levels had been altered by these therapies, our data suggests that the utility of this measurement is not significantly reduced in the presence of appropriate treatment for LVSD or ischaemic heart disease.

Our study population contained 101 patients who had a history of hypertension which on its own can increase the levels of NTproBNP [52]. In addition approximately 20% of the patients had moderate to severe mitral regurgitation and a similar proportion had a plasma creatinine level of 130 μmol/l or above, both of which are associated with elevated levels of natriuretic peptides [103]. Despite the confounding effect of all these important variables, NT-proBNP was strongly correlated to LVWMI and emerged as the strongest independent predictor of LVSD in a multivariate model. The area under the ROC curve for NT-proBNP in the diagnosis of LVSD was similar to that for BNP-32 in previous studies [45,47].

While the relationship between plasma levels of natriuretic peptides and measures of left ventricular dysfunction is well established [45,46,47,51,52,53,92], concerns have been expressed regarding the use of such measures in routine clinical practice [94]. McClure et al studied a population that may be regarded as being at high risk of LVSD i.e. survivors of AMI. These authors found that plasma BNP level was unable to differentiate between patients with mildly or moderately impaired LV function and those with preserved function. Our study on the other hand indicates a linear relationship between NTproBNP and LVWMI. Methodological differences including the method of assessment of LV function may explain these differences. In addition, while the study of McClure and the present study looked at high risk populations, differences

in the patient populations are likely to explain at least part of the difference in results.

It has been suggested that LVSD is unlikely to be present if the ECG is normal or shows only minor abnormalities [95, 104], that there is usually a major ECG abnormality in the presence of LVSD [95, 104] and that the consideration of the ECG in addition to natriuretic peptide levels can increase overall accuracy and specificity in diagnosing LVSD [93]. These findings have led to the suggestion that patients with a normal ECG are unlikely to have heart failure and therefore should not be considered for further investigation. However other studies have suggested that 10% of patients with LVSD have a normal ECG[32]. Our data indicate that consideration of the ECG as a screening tool would indeed have led to the failure to diagnose 12 patients with significant systolic impairment (LVWMI = 1.2) out of 36 with a normal ECG. A further 6 patients had LVWMI between 1.3 and 1.9. In each case plasma NT proBNP identified these patients as being likely to have LVSD. As in previous studies an abnormal ECG had low specificity in the prediction of LVSD. Moreover an abnormal ECG was not a predictor of low LVWMI on either univariate or multivariate analysis, in contrast to previous studies [93, 95]. However one study [93] did not show any association between proBNP and LV dysfunction, in contrast to the strength of association between NTproBNP and LVWMI in the current study. Further to this the other two studies [31,95] did not assess the value of natriuretic peptides in identifying LV dysfunction. One likely reason for the discrepancy is the large proportion of ECGs with a major abnormality in our study; essentially very few of the patients in this high risk population had a normal ECG.

NT-proBNP had a sensitivity of approximately 95% and specificity of 55% for the detection of LVSD. The sensitivity and specificity respectively of

various natriuretic peptides has been reported as 77% and 87% [47], 97% and 84% [46] for BNP in primary care, 91% and 72% for N-terminal BNP following MI [53]. The variability in reported sensitivity and specificity is likely to reflect differences in the selection of study populations. Of more clinical importance is the high negative predictive value (93%) of NT-proBNP in the diagnosis of LVSD in our study. This compares to previously reported values of 97% [47], 98% [46] and 97% [53].

Our study has a number of limitations. Firstly, we studied a group of patients at high risk of having LVSD and the findings may not be applicable to the identification of LVSD in a low risk group such as an unselected community population. The aim of the study was to evaluate the ability of plasma NTproBNP to identify patients with LVSD in the presence of possible confounding variables, namely drug therapy and concomitant disease. In this respect the findings of the study are unequivocal: very few patients with LVSD will not be identified by measurement of NT pro-BNP. Secondly, we did not compare the predictive value of NT proBNP to that of other natriuretic peptides such as BNP-32. However BNP-32 has been evaluated as a marker of LVSD in both a hospital population and in primary care [45,46,47,53,92]. The measurement of NT-proBNP has the advantage that it is present in quantities that are ten fold higher than BNP, potentially improving ease of detection. This would make the development of a near patient test for LVSD a distinct possibility, since existing methods for capturing and colorimetric detection of NTproBNP are capable of rapid measurement at the high plasma concentrations present in patients with heart failure.

In summary we have demonstrated in a high risk population that NTproBNP is a powerful predictor of the degree of LVSD as measured by LVWMI. NTproBNP strongly predicts LVWMI even in the presence of concomitant

drug therapy. Moreover we have shown that consideration of the ECG alone in such patients may be misleading and that consideration of the ECG in addition to plasma NTproBNP level adds little to the identification of patients with LVSD. The possibility of developing an accurate, reliable and cost effective blood test for both the diagnosis and prognostic evaluation of HF is a current challenge in the field of cardiovascular medicine. There may also be a role for the natriuretic peptides in the therapeutic monitoring of patients with HF and we have demonstrated that NT-proBNP measurements are still predictive of LVSD in treated patients. The role and cost-effectiveness of NT-proBNP as a diagnostic tool for detection of LVSD in other, particularly community based populations, needs to be established.

TABLE:1

Total patients	243
Age, median [range]	73 years[20-94]
Male	129
History of shortness of breath	132
History of Ischaemic heart disease	85
History of Hypertension	101
Clinical heart failure	102
Diuretic use	117
ACE inhibitor use	63
Digoxin use	29
Beta-blocker use	32
Creatinine, median[range]	106 μmol/L [37-316]
Wall Motion Index, median [range]	1.6 [0.2-2]

Figure 1: Correlation between NT proBNP and the left ventricular Wall Motion Index score.

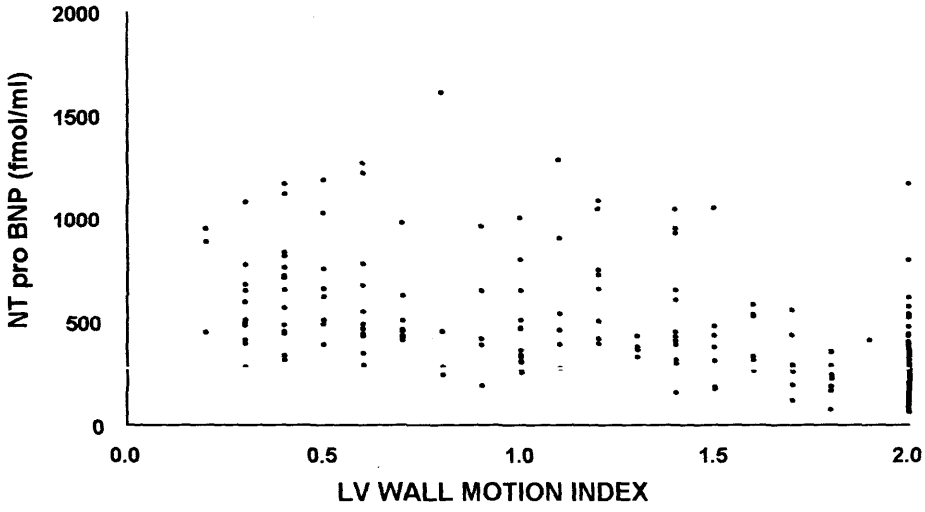


Figure 2: Plasma levels of log N-Terminal proBNP (plotted against the left ventricular wall motion index for those patients on an ACE inhibitor and/or a diuretic (Top graph) and those patients who were not on an ACE inhibitor or a diuretic (Lower graph).

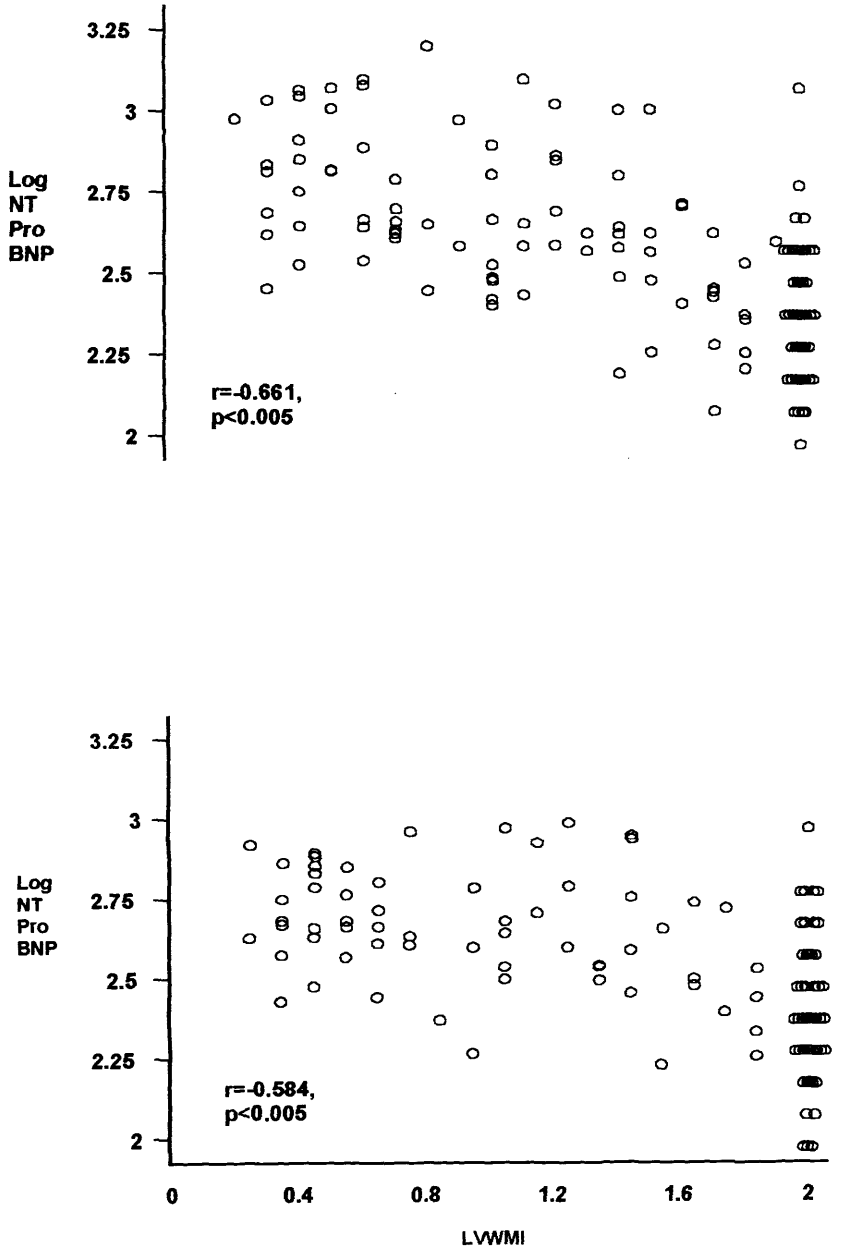


Figure 3: Correlation between Log NT proBNP and Log serum creatinine.

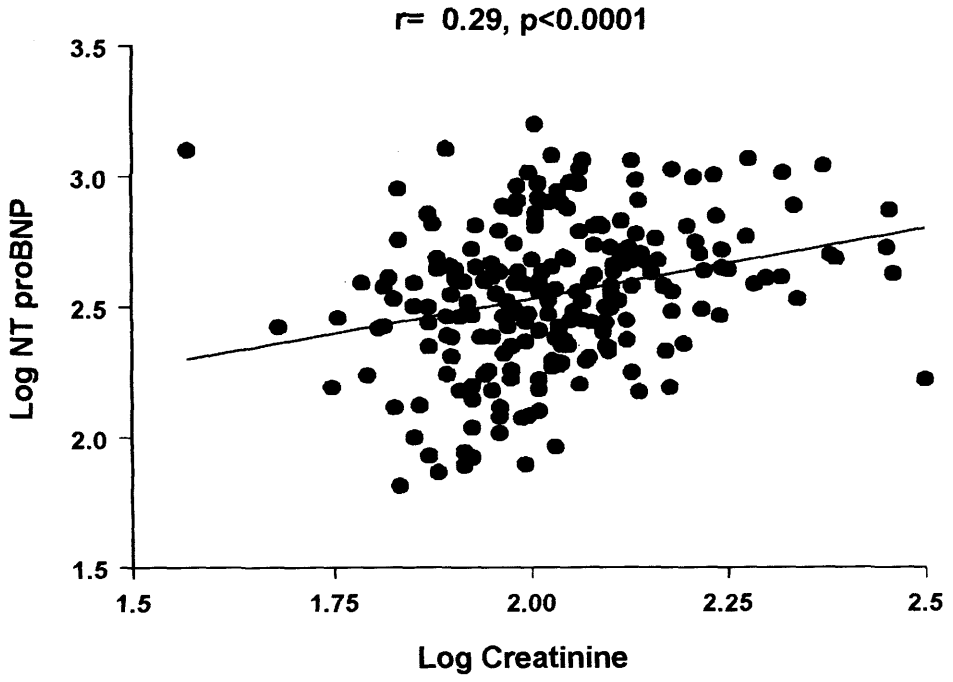


Figure 4: Correlation between Log NT proBNP and age.

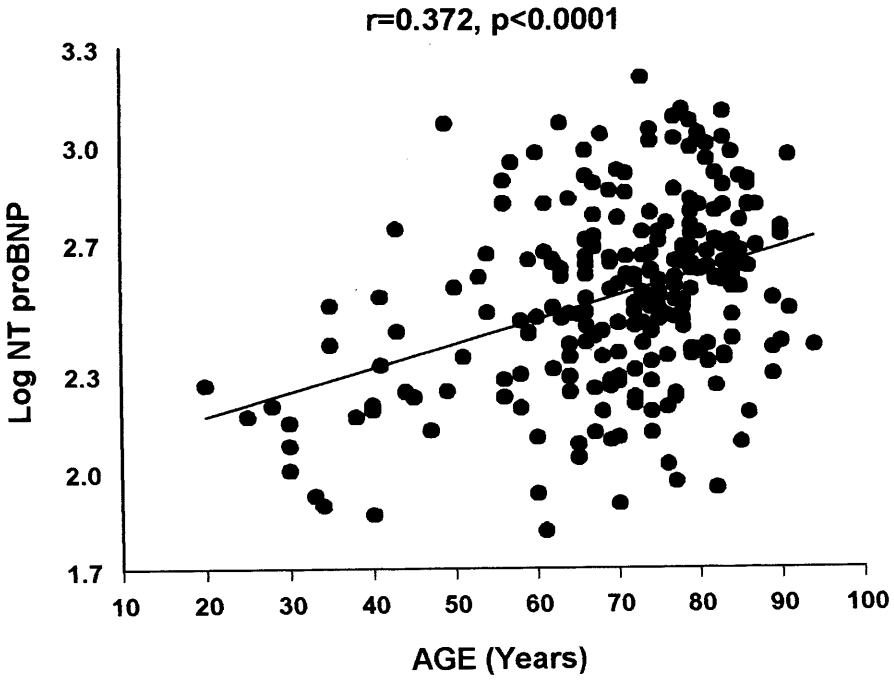


Figure 5: Box plot showing NT proBNP levels in males and females.

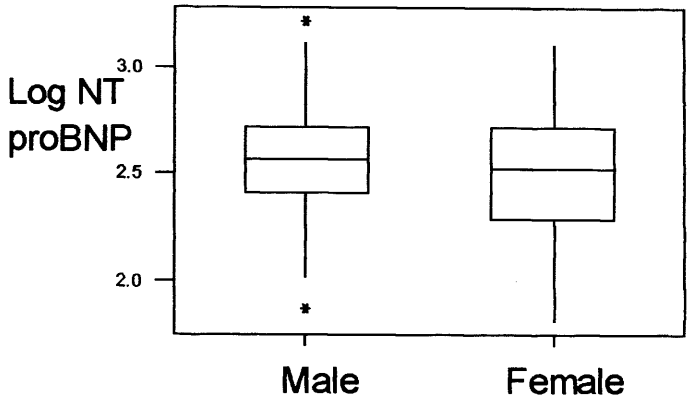


Figure 6: Boxplot showing NT proBNP levels in those patients receiving a diuretic vs. Those not receiving a diuretic.

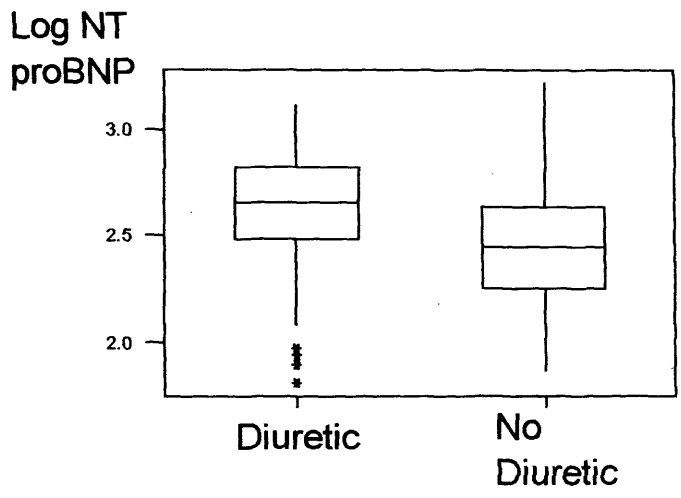


Figure 7: Box plot showing NT proBNP levels in those patients receiving an ACEI vs. Those not receiving an ACEI.

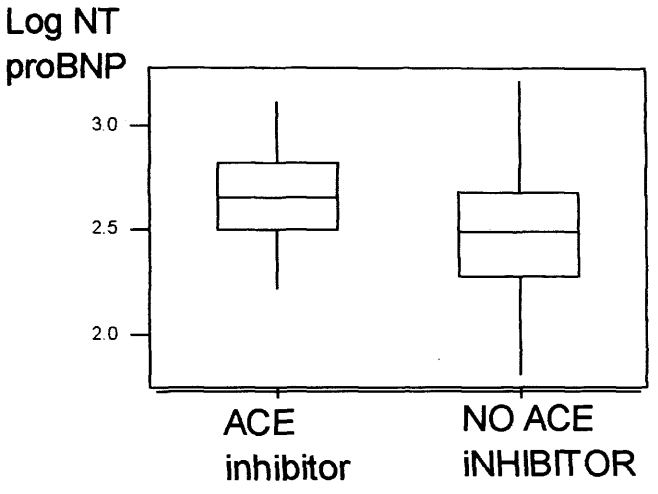


Figure 8: Box plot showing NT proBNP levels in those patients with a history of hypertension vs. Those with no history of hypertension.

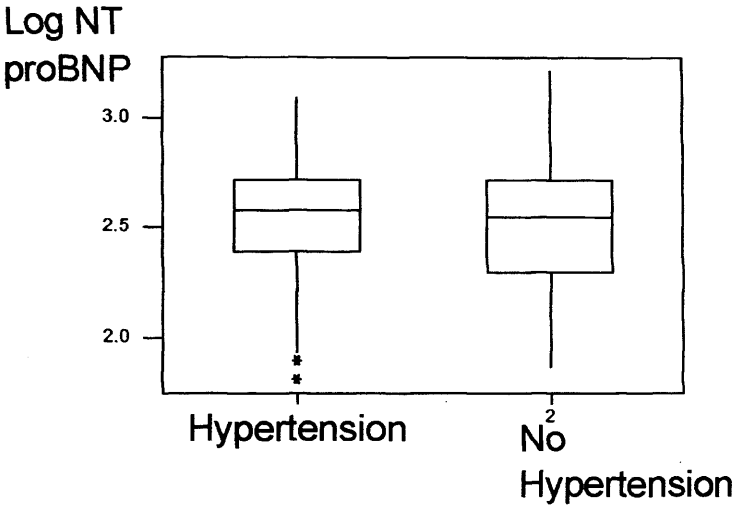


Figure 9: Box plot showing NT proBNP levels in those patients with a history of IHD vs. Those with no history of IHD.

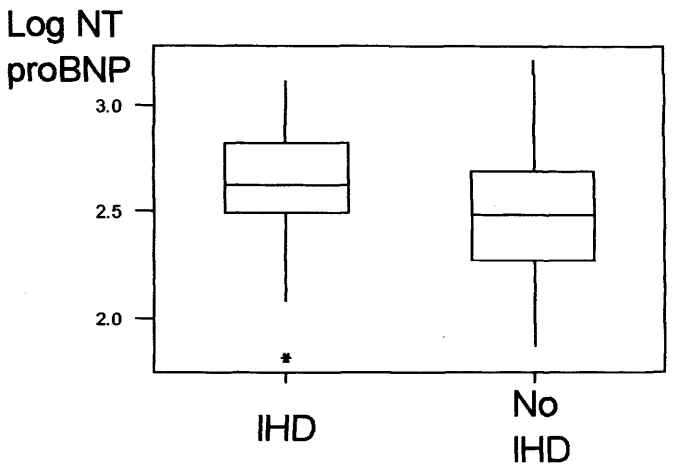


Figure 10: Box plot showing relationship of log N-Terminal proBNP levels and its relationship to the severity of mitral regurgitation.

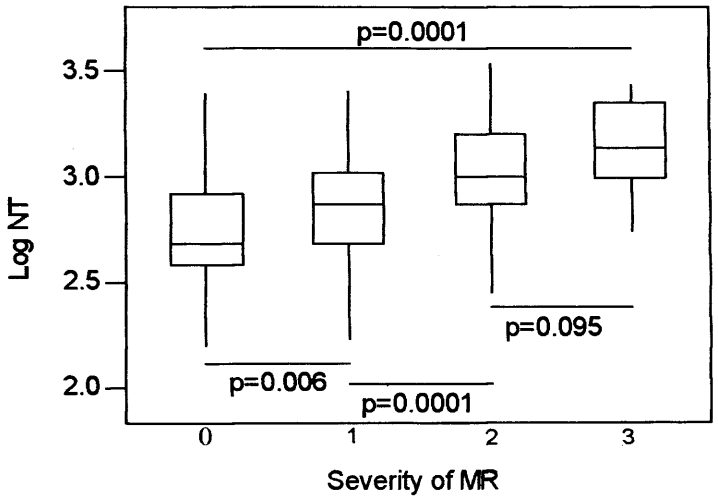
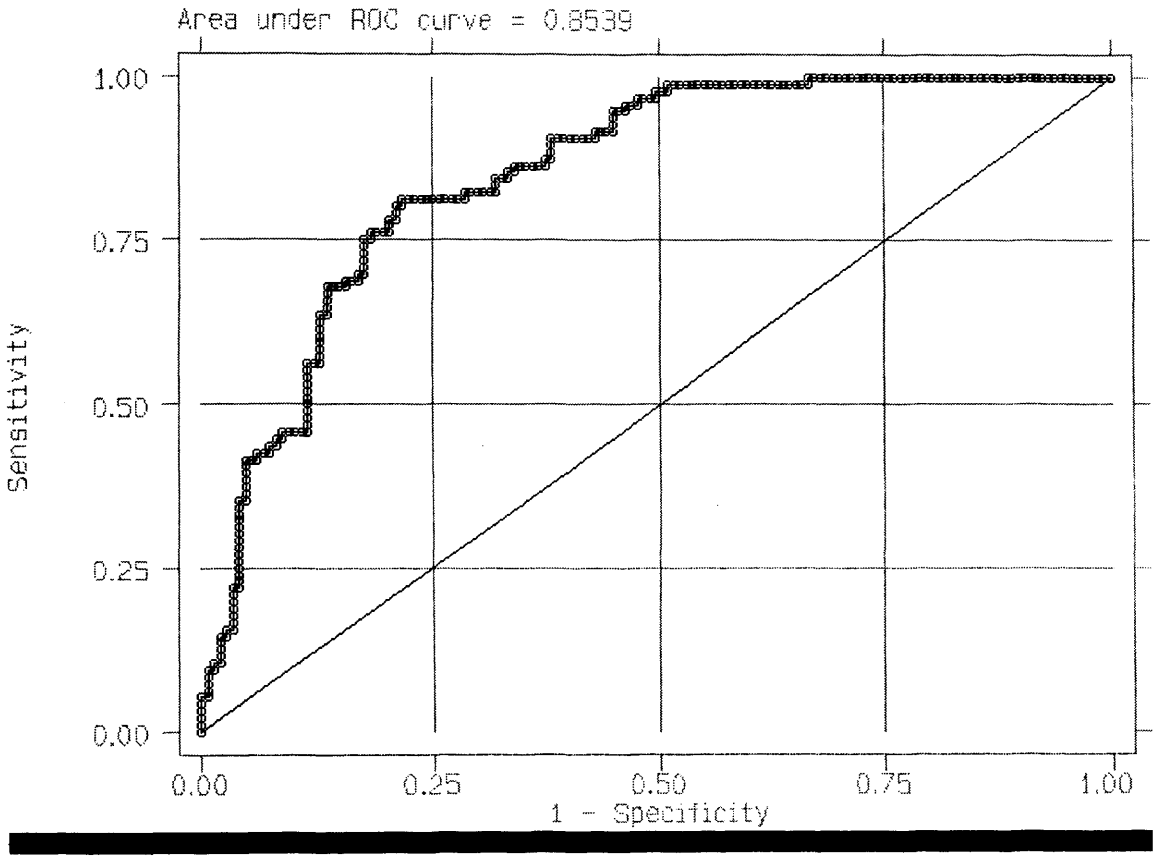


Figure 11: Receiver-Operating-Characteristic curve for ability of NTproBNP to detect left ventricular systolic dysfunction (LVWMI ≤ 1.2) in the whole study population.



CHAPTER 8

CARDIOTROPHIN-1 AND ITS RELATIONSHIP TO LVSD

INTRODUCTION

The aim of the current study was to test the diagnostic usefulness of circulating levels of CT-1 as an indicator of left ventricular systolic dysfunction (LVSD) in a high risk population referred for echocardiography. In addition we corrected for confounding factors by consideration of CT-1 as one factor in a multivariate model of the factors determining LVSD.

METHODS

Subjects

We studied 100 subjects (54 male, median age 66.5 years [20-87]) referred for echocardiography to the Cardiology Services department at Leicester Royal Infirmary. Patients were selected for study if there was (i) clinical suspicion of heart failure (n=41); (ii) history of ischaemic heart disease i.e. history of myocardial infarction, presence of pathological Q wave on the ECG, physician diagnosed angina or current use of an oral or sublingual nitrate (n=26); (iii) history of hypertension (n=39); (iv) history of shortness of breath in the absence of chronic airways disease (n=39); (v) current diuretic (n=29), angiotensin converting enzyme inhibitor (n=15) or digoxin (n=5) use.

Patients were excluded if there was a history of recent (within 1 month) acute myocardial infarction, presence of moderate or severe valvular regurgitation (i.e. mitral, aortic or tricuspid regurgitation),[8] presence of aortic or mitral stenosis or plasma creatinine $\geq$ 130 μmol/l.

Among the 100 patients we identified 10 patients, all referred with a suspected heart murmur but found to have a normal echocardiogram (normal systolic function and no significant valvular lesion). These patients (5 males, median age 52.5 years, range 40-79) were taken as echocardiographic controls. These controls had no history of shortness of breath, ischaemic heart disease or hypertension. There was no clinical evidence of heart failure in this group and they were taking no regular medication of any nature. All had serum creatinine levels less than 130 μmol/L. In addition we obtained blood samples from 10 normal healthy volunteers (5 males, median age 75.5 years, range 48-84). These individuals had no cardiovascular or respiratory history and were not on any treatment. These two groups combined (10 male, median age 62.5 years , 40-84) were used to establish the normal range of CT-1 in the study.

The study was approved by the local ethical and research committee and all subjects gave informed consent.

Echocardiography

Echocardiography was performed by a single operator (ST) using a Hewlett Packard Sonos 1500 imaging system. WMI was analysed by a single investigator (ST) blind to patient details and CT-1 results.

Immunoluminometric assay (ILMA) for CT-1

CT-1 levels were determined blind to patient details and echocardiographic findings. Each CT-1 value represents the mean of duplicate measurements.

Statistics

Concentrations of CT-1, WMI and age of the patients were not normally distributed and were log transformed before analysis. Results are expressed as medians [ranges] and compared between groups by ANOVA followed by student's T-test for unpaired data where appropriate. Pearson's correlation coefficients were also computed. Predictive models for the response variable (Log WMI) were developed using multiple linear regression analysis. All statistical analyses were carried out using the software package Minitab (Minitab Inc., PA, USA). Two tailed p values under 0.05 were considered significant.

RESULTS

The characteristics of the study population (100 subjects) are shown in Table 1.

Concentration of CT-1 (median, [range] in fmol/ml) was 34.2 [6.9-59.7] in the controls (Group 0). Subjects with WMI =2 (Group1), WMI 1.9-1.3 (Group 2) and WMI = 1.2 (Group 3) had CT-1 concentrations of 53.9 [18.23-120.8), 48.8 [21.7-99.6] and 75.5 [31.4-130.6] respectively (Figure 1) ($p<0.0001$, ANOVA).

Mean log CT-1 was higher in each of the groups 1, 2 and 3 as compared to group 0 ($p=0.005$, $p=0.02$ and $p<0.0001$ respectively). Furthermore mean log CT-1 was higher in group 3 when compared to group 1 ($p=0.005$) and

group 2 (p=0.02). There was no significant difference of log CT-1 between group 1 and 2 (p=0.71).

Mean log ages differed amongst the groups (p=0.03). Patients in group 1 were younger than in group 2 (p=0.002) and group 3 (p=0.004). Mean log ages were similar in groups 1, 2 and 3 as compared to group 0 (p=0.3, p=0.1 and p=0.2 respectively). There was no significant difference of log age between group 2 and 3 (p=0.81).

Log CT-1 was correlated to the presence of clinical left ventricular failure (r=0.21, p<0.05) and log WMI (r= -0.21, p<0.05).

We considered log age, log CT-1, gender, history of shortness of breath, history of IHD or hypertension, current diuretic or ACE inhibitor use and serum creatinine in a multivariate model of the predictors of log WMI. The only significant predictors were use of ACE inhibitor (P=0<0.005) and log CT-1(P<0.05). On best subsets analysis the strongest correlate with log WMI was use of ACE inhibitor (R^2=16.7%). The addition of log CT-1 improved diagnostic accuracy (R^2=20.1%,p<0.05).

DISCUSSION

We have demonstrated increased circulating plasma CT-1 in a high risk population of patients referred for echocardiography. We have demonstrated for the first time a significant correlation between CT-1 concentration and WMI. The highest concentration of CT-1 was seen in those patients with significant LVSD i.e. WMI $\leq$ 1.2. Furthermore we have shown plasma CT-1 concentrations to be a weak but significant predictor of WMI in a multivariate regression model.

Although CT-1 concentration was highest in the group with a WMI of $\leq$ 1.2 it was also raised in the group of patients with a WMI of 2 when compared to normal controls. The possible explanation being that although this group had a WMI of 2 they were not normal subjects. Most of them were in-patients and had a some degree of cardiovascular morbidity.

We excluded patients with significant valvular lesions because conditions such as aortic stenosis increase systolic wall stress [105] and in situations of volume overload (such as mitral regurgitation) a similar form of eccentric hypertrophy occurs with time[106]. Also patients with a recent myocardial infarction were excluded because augmented ventricular gene expression of CT-1 following myocardial infarction in rats has been demonstrated[107]. Patients with known renal dysfunction were also excluded from this study as it is currently not known if CT-1 levels are affected by renal dysfunction.

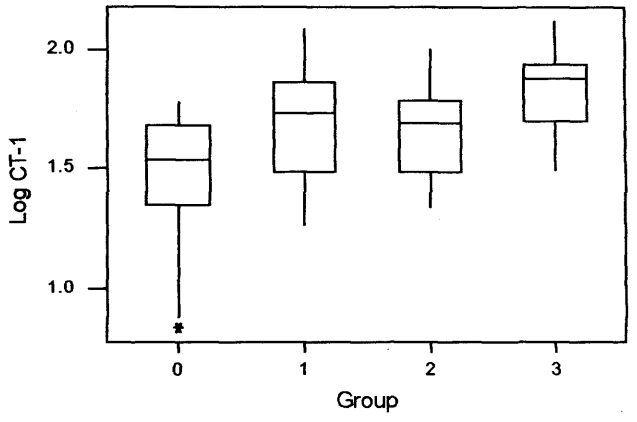
Our study has a number of limitations. Although we have demonstrated a correlation between CT-1 and LVSD we did not assess ventricular volumes and wall thickness. It is certainly possible that the subjects with the highest CT-1 levels could be those who had evidence of ventricular dilatation and cardiac remodeling. Furthermore we did not assess the presence of diastolic dysfunction. We also did not measure other circulating cytokines in the study such as interleukin-6 and tumor necrosis factor which have been shown to contribute to the process of ventricular remodelling and in addition provide additional prognostic information.

Our data suggests there may be a relation between heart failure severity and CT-1 concentration. Additional studies are needed to further evaluate the role of CT-1 in heart failure. Whether the high levels of CT-1 can provide prognostic information in heart failure, independent of left ventricular systolic function is not known.

Table 1: Characteristics of the study population (100 subjects).

	Group 1, WMI=2	**Group 2, WMI 1.9-1.3**	**Group 3, WMI<1.2**
	(n=62)	**(n=15)**	**(n=23)**
Median WMI[Range]	2	1.7[1.4-1.8]	0.7[0.3-1.2]
Male	28(45%)	11(73%)	15(65%)
Age(yrs), median [range]	64[20-85]	71[56-85]	67[43-87]
History of SOB	15(24%)	8(53%)	16(70%)
History of IHD	13(21%)	3(20%)	10(43%)
History of HT	17(27%)	9(60%)	13(57%)
Clinical LVF	3(5%)	5(33%)	15(65%)
Diuretic	14(23%)	5(33%)	10(43%)
ACE Inhibitor	4(6%)	1(7%)	10(43%)

Figure 1: Box plot showing relationship of log CT-1 levels and its relationship to the severity of LVSD. Group 0 =normal controls, Group 1= WMI of 2, Group 2 =WMI 1.3 to 1.9 and Group 3 = WMI $\leq$ 1.2.



CHAPTER 9

The effect of valvular regurgitation on plasma Cardiotrophin-1 in patients with normal left ventricular systolic function

INTRODUCTION

The mechanisms involved in the development of acute heart failure after an acute myocardial infarction are easy to understand. In contrast, the mechanisms that lead a patient from asymptomatic cardiac dysfunction (valvular or ischaemic) to chronic heart failure are still unknown. The mechanical stimuli of volume overload promotes an eccentric form of hypertrophy and ventricular dilatation with time. On a single cell level, cardiac myocytes isolated from such dilated hearts exhibit an increase in cell length, reflecting the addition of sarcomeric units in series [108,109]. This hypertrophy induced by volume overload eventually results in an irreversible loss of function.

We investigated the potential role of CT-1 in ventricular dilatation due to valvular regurgitation. We hypothesized that plasma CT-1 would be raised in patients with significant mitral, tricuspid and/or aortic regurgitation (MR/TR/AR respectively) and normal left ventricular (LV) systolic function when compared to those with no (or mild) MR/TR/AR and normal LV systolic function.

METHODS

Subjects

We studied seventy eight patients (30 male, median age 63.5 years, range 20-89), all with normal LV systolic function (Wall motion index of 2) who were referred to our echocardiography department. All patients with aortic stenosis, mitral stenosis, recent myocardial infarction (within last 4 weeks) or renal impairment (plasma creatinine $\geq$ 140 μmol/L) were not included in the study.

Echocardiography

Echocardiography was performed by a single operator (ST) (blind to patient details and the CT-1 results) using a Hewlett Packard Sonos 1500 imaging system. Color flow Doppler on the parasternal, apical 4 chamber and the apical long axis views was used to semi-quantify MR, TR and AR. Regurgitation was graded as absent/trace/mild=1 or moderate/severe= 2 based upon the evaluation of the regurgitant jet area (i.e. regurgitation jet velocity).

Immunoluminometric assay (ILMA) for CT-1

CT-1 levels were determined blind to patient details and echocardiographic findings. Each CT-1 value represents the mean of duplicate measurements.

Statistics

Concentrations of CT-1, creatinine and age of the patients were not normally distributed and were log transformed before analysis. Results are expressed as medians [ranges] and compared between groups by ANOVA followed by student's T-test for unpaired data where appropriate. Pearson's correlation coefficients were also computed. Predictive models for the response variable (MR) were developed using multiple regression analysis.

All statistical analyses were carried out using the software package Minitab (Minitab Inc., PA, USA). Two tailed p values under 0.05 were considered significant.

RESULTS

The characteristics of the study population (n=78) are shown in Table 1. The subjects with a regurgitant lesion were older ($p<0.001$), were more likely to have a history of hypertension ($p=0.02$) and were more likely to be on a diuretic ($p=0.02$).

Sixty three subjects had no significant valvular lesion (although it should be noted these are not normal controls as a significant number of these subjects have IHD or a history of hypertension-table 1). Seven subjects had moderate/severe MR, 9 had moderate/severe TR and 4 had moderate/severe AR. These subjects had CT-1 concentrations (reported as medians in fmol/ml, [range]) of 51.6[18.2-114.5], 83.8[41.5-175.3], 71.1[32.9-175.3] and 46.7[21.2-79.2] respectively (Figure 1) ($p=0.02$, ANOVA).

Mean log CT-1 was higher in those with moderate/severe MR when compared to those without a significant regurgitant valvular lesion (denoted as group 0 in figure 1, $p=0.03$). Although patients with moderate/severe TR had a trend towards higher CT-1 levels when compared to the group without significant valvular lesions, this did not achieve conventional levels of statistical significance ($p=0.2$). There was no difference in CT-1 levels between those with moderate/severe AR vs. group 0 ($p=0.8$).

There was a significant correlation between the degree of MR present and log CT-1 concentration (r=0.32, p=0.004). In a multivariate model to predict the degree of MR we considered log age, gender, history of IHD and HT, current diuretic or ACE inhibitor use and log CT-1. The only significant predictor of the degree of MR was log CT-1 (P=0.004). The model was significant with r^2 =16% (p=0.006).

Discussion

We have demonstrated for the first time significant elevation of CT-1 in subjects with moderate/severe MR despite them having normal LV systolic function. We were unable to demonstrate a similar increase in AR. The likely reason for this is the small number of subjects with moderate/severe AR.

The normal LV systolic performance noted in subjects with significant MR may be due to the continuous addition of sarcomeres in series in order to maintain LV function despite an increased radius of curvature. These alterations in the physical properties of the ventricular wall might be the cause rather than the consequence of the progression to heart failure in some of these patients. If this hypothesis is correct, it raises an important therapeutic question, could pharmacological manipulation of the CT-1 receptor have therapeutic potential in the protection of cardiac failure ?

Patients with valvular regurgitant lesions are often referred for surgery only after irreversible LV dysfunction has developed. Whether plasma CT-1 concentrations could serve as a marker of early LV dysfunction cannot be

answered from this cross-sectional study and needs to be tested prospectively. Changes in CT-1 levels may reflect early changes in ventricular physiology which occur in the early part of the disease process before they can be detected echocardiographically.

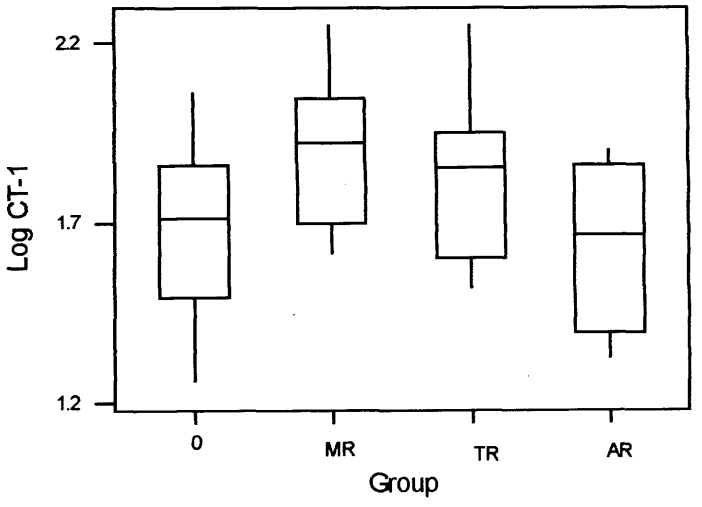
The main limitation in our study is the small number of patients with moderate/severe regurgitant lesions who had normal left ventricular systolic function. Such patients are not common since many would develop systolic dysfunction with such a degree of mitral regurgitation. Another limitation is that the subjects with significant regurgitant lesions were older and were more likely to have hypertension. In a multivariate model the only significant predictor of the degree of MR was log CT-1 and not age or a history of hypertension. Furthermore it was not possible to assess ventricular volumes and wall thickness in all patients in this study. Regarding the WMI, scoring of each segment is a subjective estimate, but the method has been shown to be reproducible [79].

Despite years of research, the mechanisms by which changes in hemodynamic load are translated into myocardial hypertrophy are not fully understood. This study suggests for the first time that one possibility could be an elevation of CT-1 levels that would over time produce an eccentric hypertrophic phenotypic response and morphometric changes at the myocyte level characteristic of those seen in conditions of volume overload. CT-1 may thus be responsible for the left ventricular contractile dysfunction seen over a period of time in patients with MR. The possibility of using such a marker to select those patients at most urgent need for surgical intervention remains to be tested.

TABLE1:

	Regurgitation(MR,TR and/or AR)	**No valvular regurgitation**
	n=15	n=63
Age,Median[range]	78[54-89]	65[20-86]
Males	2(13%)	28(44%)
History of IHD	2(13%)	14(22%)
History if hypertension	10(67%)	17(27%)
On diuretic	9(60%)	14(22%)
On ACE inhibitor	1(7%)	5(8%)

Figure 1: Box plot showing relationship between log CT-1 levels and the presence of a significant regurgitation lesion (MR/TR/AR). Group 0= those without a significant regurgitant valvular lesion.



CHAPTER 10

Plasma NT pro BNP and Cardiotrophin-1 in aortic stenosis

INTRODUCTION

Aortic stenosis (AS) is usually an idiopathic disease resulting from degeneration and calcification of the aortic valve leaflets [110], the incidence and prevalence of which may be expected to rise as the number of elderly individuals in the population increases. Echocardiography and Doppler examination are essential for the accurate diagnosis and quantification of the severity of the stenosis. Serial examinations are required to monitor the progression of the condition and determine the optimal time for corrective surgery.

BNP has previously been shown to correlate with left ventricular end-systolic wall stress in patients with AS [111]. The relationship of plasma concentrations of NT-proBNP to the severity of AS are not known in patients with aortic stenosis.

In addition, CT-1 which is a cardiac myocyte hypertrophy inducing factor has been shown to stimulate BNP production at a transcriptional level from cultured neonatal rat cardiomyocytes [66], and raises the possibility that CT-1 is involved in the secretion of BNP clinically observed in severe cardiac hypertrophy.

Since the plasma assays for these cardiac peptides are relatively cheap, non-invasive and not operator dependent, we examined their utility for

monitoring patients with AS and investigated their correlation to the maximum trans-valvular aortic pressure gradient (TVPG).

METHODS

Subjects

We studied fifteen patients (5 male, mean age 79 years, range 60-94), all with AS (mean TVPG 39.3 mm Hg, range 20-100) who were referred to our echocardiography department. All patients with LVSD, mitral stenosis, moderate or severe mitral regurgitation on color Doppler or a recent myocardial infarction (within last 4 weeks) were not included in the study.

In addition 5 elderly echocardiographic normal controls (3 male, mean age 68.6 years, range 56-79) and 5 elderly normal healthy volunteers (2 male, mean age 67.4 years, range 60-79) were studied. These 2 groups were combined (5 male, mean age 68 years, range 56-79) to obtain the normal range for NT proBNP and CT-1 in this study.

Echocardiography

Echocardiography was performed using a Hewlett Packard Sonos 1500 imaging system and recordings were on Panasonic Super VHS tapes. Continuous wave Doppler was performed using a 1.9 MHz non-imaging transducer. Recordings of the aortic valve systolic velocities were performed in the apical, right parasternal and suprasternal positions. The TVPG was obtained from the peak aortic Doppler velocity using the modified Bernoulli equation [112].

Immunoluminometric assays for NT proBNP & CT-1

NTproBNP and CT-1 levels were determined blind to patient details. Each NTproBNP and CT-1 value represents the mean of duplicate measurements.

Statistics

All statistical analyses were carried out using the software package Minitab (Minitab Inc, PA, USA).The TVPG levels were not normally distributed and were log transformed before analysis. All results are expressed as mean [ranges] and comparisons were by the Mann Whitney test. Pearson's correlation coefficients were computed. Comparisons with $p<0.05$ were considered significant. All results are expressed as mean [ranges] in fmol/ml.

RESULTS

In the study (n=15), 2 subjects gave a history of ischaemic heart disease (IHD) and 9 gave a history of hypertension (HT). Six subjects were on a diuretic, 4 on an angiotensin converting enzyme inhibitor, 1 on digoxin and 1 on a beta-blocker. None of the subjects had severe aortic or tricuspid regurgitation on color flow Doppler examination.

The subjects with AS were older than the controls (mean age 79 years [range 60-94] vs. mean age 68 years [range 56-79], p=0.01).

NT proBNP levels were elevated in the subjects with AS as compared to the normal controls (252.9 [79.2-541.8] vs. 157.2 [104.7-236.9] fmol/ml, $p<0.005$){Figure 1}. Also CT-1 was elevated in the AS group when compared to the controls (57.3 [33-86.3] vs. 28.3 [6.9-48.3] fmol/ml, $p<0.0005$){Figure 2}.

Both NT proBNP and CT-1 levels correlated to the TVPG ($r=0.53$ & $r=0.65$, $p<0.05$ & $p=0.009$, respectively, figures 3 and 4). Furthermore NT proBNP and CT-1 levels in the AS patients were weakly correlated ($r=0.49$, $p=0.06$).

There was no significant correlation between the NT proBNP and CT-1 levels with the age of the patient ($r=0.14$, $r=0.11$, both not significant).

We considered NT proBNP, CT-1, age and gender of the patient, history of IHD and HT and WMI in a multivariate model of the predictors of the TVPG. The model was significant with an R^2 of 60%, the significant predictors of the TVPG were CT-1 and history of IHD. On best subset analysis CT-1 alone had an R^2 of 38% and in combination with a history of IHD this improved to 49%. NT proBNP alone had an R^2 of 22% and when combined to that of CT-1 did not improve the diagnostic accuracy (R^2 =39%).

DISCUSSION

We have demonstrated for the first time increased plasma concentrations of both NT proBNP and CT-1 in subjects with AS when compared to elderly controls. We have also shown that both the peptide concentrations correlated to the severity of AS, although in a multivariate model only CT-1 came out to be the most significant predictor of the severity of AS. The strong correlations of the peptide levels to the severity of AS suggest their use as additional non-invasive investigations in monitoring disease progression, although this would need to be tested by a prospective study. In addition, the role that CT-1 may play in the ventricular dilatation of decompensated AS is unknown but the study suggests a potential therapeutic target for further study.

NT proBNP seems likely to be elevated in subjects with AS because of the increased left ventricular end-systolic wall stress observed in these patients [111]. The mechanism for the elevated CT-1 in subjects with AS remains unclear. However, increased wall stress, diastolic dysfunction, left ventricular hypertrophy and ischaemia may all be factors capable of stimulating CT-1 release from myocytes.

Subjects with LVSD and a recent MI were excluded from this study. This is because NT proBNP is known to be elevated in these subjects [53]. Furthermore there is also evidence to suggest that CT-1 may be elevated following a MI and be involved in the process of ventricular remodelling [107].

The major limitation in the study was that the control population was not age matched for the AS subjects. Although the controls were elderly subjects (mean age 68 years) the subjects with AS were older (mean age 79 years). Another limitation was the small size of the study population.

The precise mechanism by which plasma concentrations of CT-1 are raised in subjects with AS remain to be determined. Whether these novel cardiac derived peptides could be used to monitor disease progression or be used in cardiovascular screening programs remains to be investigated further.

Figure 1: Plasma levels of NT-proBNP in control subjects and patients with aortic stenosis. The means are shown in solid bars.

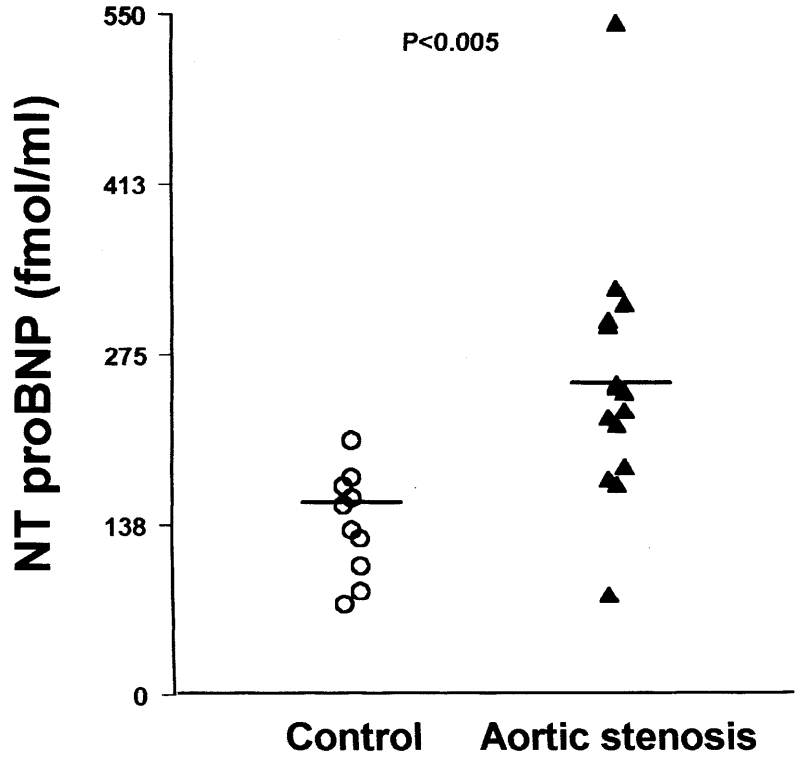


Figure 2: Plasma levels of CT-1 in control subjects and patients with aortic stenosis. The means are shown in solid bars.

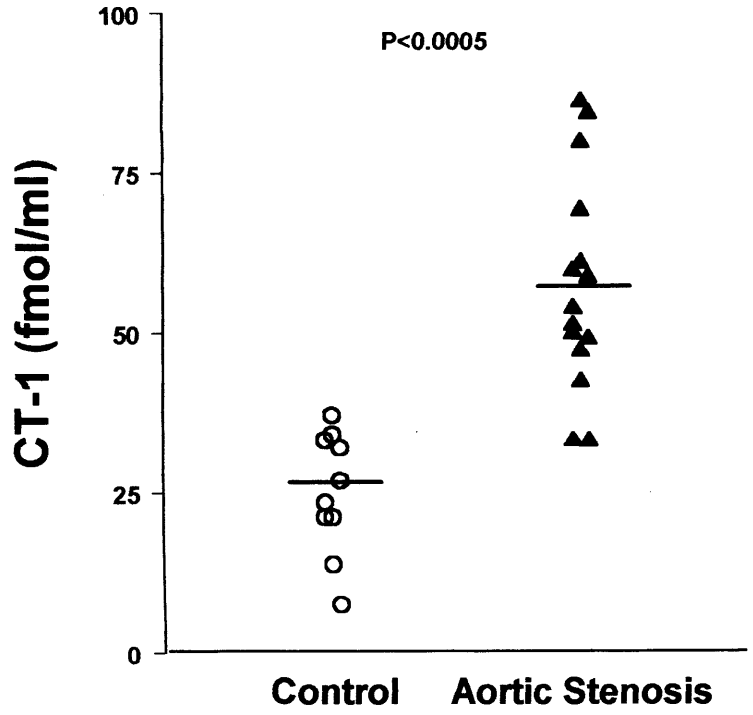


Figure 3: Correlation between NT-proBNP and log maximum trans-valvular aortic pressure gradient (TVPG) in the subjects with aortic stenosis.

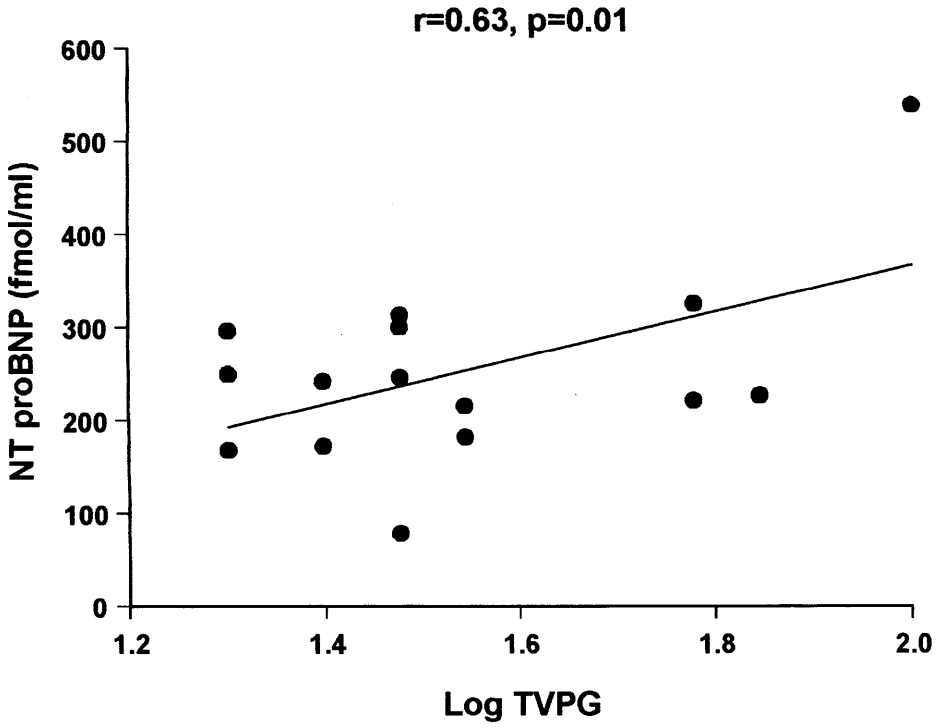
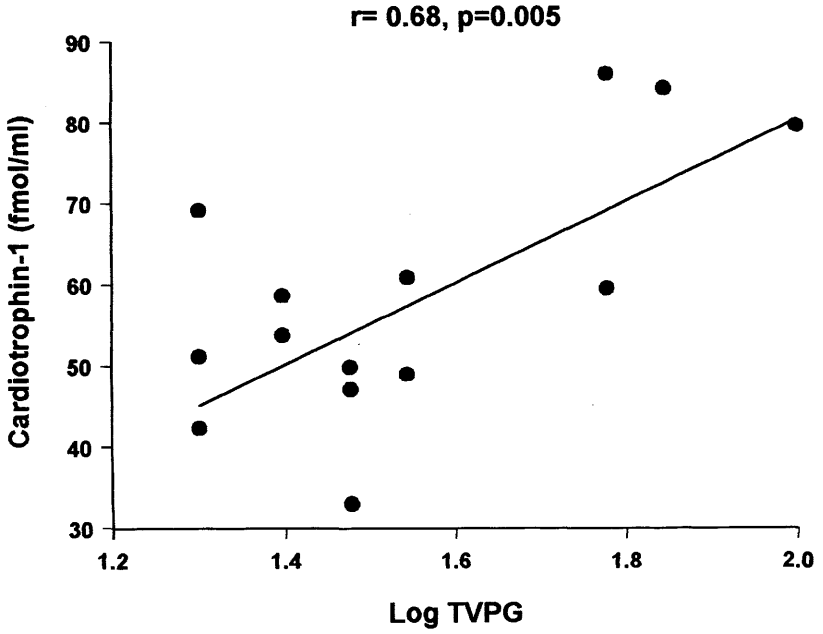


Figure 4: Correlation between CT-1 and log maximum trans-valvular aortic pressure gradient (TVPG) in the subjects with aortic stenosis.



CHAPTER 11

NT proBNP Identifies Patients With A Low Wall Motion Index Following Acute Myocardial Infarction

INTRODUCTION

Recent interest has focused on the use of neurohormonal markers such as BNP, ANP, NT ANP and NT PROBNP as indices of LVSD and prognosis following AMI [37,41,53]. The secretion of these neurohormones may reflect not just existing LVSD but may in addition be a sensitive index of abnormal wall stress preceding the process of ventricular remodelling [39,113].

A recent study has shown that plasma levels of NT PROBNP measured 2 to 4 days after AMI independently predicts left ventricular ejection fraction (LVEF) and 2-year survival [53]. The correlation of LVEF with NT PROBNP levels obtained in this study were comparable with those observed for BNP and clearly stronger than those observed for ANP and NT ANP [53].

The optimum time of sampling for NT PROBNP following AMI has not previously been investigated. Furthermore, temporal patterns of secretion of NT PROBNP may differ in those with and without LVSD after AMI, as has been demonstrated for BNP [39]. The aims of this prospective study were to identify the optimum time of sampling for NT PROBNP following AMI and to examine the potential use of NT PROBNP as a marker for LVSD as assessed echocardiographically. In addition we examined the potential use of NT PROBNP as a marker of ventricular remodelling (LV

dilatation or development of significant mitral regurgitation), diastolic dysfunction or death post AMI.

METHODS

SUBJECTS

We measured NT PROBNP in 60 patients (45 Male, median age 63.5 years, range 36-87, 39 anterior AMI) admitted to the Coronary Care Unit of Leicester Royal Infirmary with a diagnosis of AMI. Serial blood samples were taken at 14-48 hours, 49-72 hours, 73-120 hours, 121-192 hours following AMI and at an out patient (OP) visit in the survivors.

Echocardiographic assessment of Wall Motion Index (WMI), a measure of left ventricular dysfunction, was made in 58 of the 60 patients during admission (WMI-1;Median day 4.5, range 2-6) and at the OP visit (WMI-2;Median day 50, range 20-73) in 52/54 survivors.

The study was approved by the local ethical and research committee and all subjects gave written informed consent.

ECHOCARDIOGRAPHY

WMI was calculated using a nine-segment model [78,79] and analysed by a single investigator (ST) blind to patient details and NT PROBNP results.

Mitral regurgitation (MR) was graded as absent=0, trace=1, moderate=2 or severe=3 on color flow Doppler analysis based on the area of the jet projecting into the left atrium [96].

The automated boundary detection technique was applied to measure left ventricular end systolic volume (ESV) and end diastolic volume (EDV) online from the apical 4 chamber view [114]. Segments in which endocardial motion was not well defined by the automated boundary detection technique were prospectively excluded from quantitative analysis.

Transmitral flow recordings were obtained in quiet expiration from the tips of the mitral valve leaflet. The following Doppler indices were measured: Peak early (E) and atrial (A) transmitral filling velocities, E/A ratio and E-deceleration time (DT).

BLOOD SAMPLES

A 20 ml sample of venous blood was taken at each of 5 different time periods (as above) following the index AMI. Blood was transferred into pre-chilled EDTA (1.5 mg/ml blood) tubes containing 500 IU/ml of aprotinin. Samples were immediately centrifuged at 4°C and plasma separated and then stored at -70°C until assayed.

IMMUNOLUMINOMETRIC ASSAY FOR NT PROBNP

The methodology for assay of NT PROBNP has been described previously [89]. NT PROBNP levels were determined blind to patient details. Each NT PROBNP value represents the mean of duplicate measurements. The normal range for NT PROBNP in our laboratory is < 200 fmol/ml.

STATISTICAL ANALYSIS

Assessment was made of the strength of relationship between left ventricular systolic function during and after hospitalisation and NT PROBNP measured at the various time intervals. The relationship with WMI of a number of clinical and laboratory variables in addition to NT PROBNP was investigated. Predictive models for the response variable (WMI) were developed using multiple linear regression analysis and stepwise logistic regression analysis. In addition we investigated the relationship between NT PROBNP with outcome and with left ventricular remodelling assessed using the change in echocardiographic parameters between hospitalization (WMI-1, EDV-1 and ESV-1) and following discharge (WMI-2, EDV-2 and ESV-2).

Concentrations of NT PROBNP, age, plasma creatinine, plasma glucose on admission, peak creatine kinase (CK), EDV from scan 2 (EDV2), ESV from scan 2 (ESV2) and WMI scores were not normally distributed and were log transformed before analysis. All statistical analyses were carried out using the software package Minitab (Minitab Inc, PA, USA). All comparisons were by the students t test for unpaired data. Pearson's correlation coefficients were computed. Comparisons with $p<0.05$ were considered significant. All results are expressed as means $\pm$ SEM (fmol/ml).

RESULTS

Demographic and outcome data for the 60 patients is presented in table 1. Biochemical and echocardiographic data for the total population is in table 2.

Compared to the normal range (<200 fmol/ml) NT PROBNP levels were elevated at 14-48h (748 $\pm$ 170), 49-72h (579 $\pm$ 138), 73-120h (450 $\pm$ 124), 121-192 h (823 $\pm$ 257) and at the OP visit (807 $\pm$ 176). There was a

significant drop in NT PROBNP levels from 14-48 hours to 73-120 hours ($p=0.007$), following which there was a significant increase in NT PROBNP levels at the OP visit ($p=0.01$).

Mean NT PROBNP levels in anterior MI were higher at all time points compared to inferior MI (1000 ± 255 vs 292.8 ± 41.1, $p<0.005$ at 14-48 h, 732 ± 199 vs 261.4 ± 44.6, $p<0.05$ at 49-72 h, 595 ± 196 vs 214.1 ± 29.5, $p<0.005$ at 73-120 h, 1140 ± 374 vs 187.4 ± 22.3, $p<0.005$ at 121-192 h and 1162 ± 268 vs 246.4 ± 40.9, $p<0.005$ at the OP visit).

In patients who had suffered anterior AMI there was a significant drop in NT PROBNP levels between 14-48 hours and 73-120 hours ($p<0.05$), following which there was a significant increase in NT PROBNP levels at the OP visit ($p=0.007$) {Figure 1}. This biphasic response was not seen following inferior AMI. In the group of patients with inferior AMI NT PROBNP levels did not differ significantly among time points ($p=0.6$). {Figure 1}.

In the population as a whole WMI-1 correlated with NT PROBNP at 14-48h ($r= - 0.52$, $p<0.005$), 49-72h ($r= - 0.57$, $p<0.005$), 73-120h ($r= - 0.64$, $p<0.005$) and 121-192 h ($r= - 0.57$, $p<0.005$) {Figures 2-5}.

The correlation of WMI-2 with NT PROBNP was less strong: at 14-48h ($r= - 0.29$, $p<0.05$), 49-72h ($r= - 0.26$, $p=0.09$ NS), 73-120h ($r= - 0.41$, $p=0.005$) and 121-192h ($r= - 21$, $p=0.18$ NS) {Figures 6,7}. WMI-1 and WMI-2 correlated strongly ($r= 0.756$, $p<0.0001$).

NT PROBNP at 14-48 hours correlated with NT PROBNP levels at 49-72 hours ($r= 0.57$, $p<0.0001$), 73-120 hours ($r= 0.68$, $p<0.0001$), 121-192

hours (r= 0.56, p<0.0001) and NT PROBNP levels at the OP visit (r= 0.31, p<0.05).

There was no significant correlation of plasma creatinine on admission with NT PROBNP levels at any time following AMI.

We considered NT PROBNP at 14-48h, 49-72h, 73-120 h, 121-192h, age, gender, ethnicity, past history of AMI, history of hypertension, history of diabetes mellitus, ECG site of infarct, plasma creatinine on admission and radiological heart failure in multivariate models for the predictors of WMI-1. On best subsets analysis the strongest correlate with WMI-1 was NT PROBNP at 73-120h (R^2=39%, p<0.005). The addition of ECG site of infarct improved diagnostic accuracy (R^2=49%, p<0.005).The other significant predictors of WMI-1 in the model were history of diabetes mellitus (p<0.05) and previous history of AMI (p<0.05), which when combined with NT PROBNP at 73-120h and ECG site of infarct increased R^2=58%, p<0.005.

We considered the same variables in a multivariate model for the predictors of WMI-2. On best subsets analysis the strongest correlate with WMI-2 was NT PROBNP at 73-120h (R^2=15%). The addition of ECG site of infarct improved diagnostic accuracy (R^2=20%, p<0.05).The only other significant predictor of WMI-2 in the model was a past history of AMI which improved diagnostic accuracy of the model to R^2=26%, p<0.005.

NT PROBNP level at 73-120h (R^2=12.5%, p<0.05) and age of the patient (R^2=15.6%, p<0.05) were the only significant predictors of poor outcome (LVSD / significant MR / apical thrombus / death / ventricular remodelling (defined as an increase in ESV2 of 30ml or EDV2 of 50ml compared to

volume measurements on scan 1)) in a multivariate model (R^2=21%, p<0.05).

NT PROBNP > 240 fmol/ml at 73-120h had a positive predictive value of 74% and a negative predictive value of 61% in predicting WMI-1 <1.2. NT PROBNP > 240fmol/ml at 73-120h had a positive predictive value of 41% and a negative predictive value if 91% in predicting WMI-2 < 1.2 or death.

NT PROBNP > 500 fmol/ml at any time point during the hospital stay had a positive predictive value of 47% and a negative predictive value of 100% in predicting death or WMI-2 of $\leq$1.2.

There was no significant correlation between ESV-2 and EDV-2 in the survivors and NT PROBNP levels. However EDV-2 correlated weakly with NT PROBNP at 14-48 hours (r=0.28, p=0.06 NS) and 121-192 hours (r=0.3, p=0.09 NS). ESV-2 correlated with NT PROBNP at 14-48 hours (r=0.27, p=0.08 NS) and NT PROBNP at 121-192 hours (r=0.26, p=0.1 NS).

There was a significant correlation between the change in ESV from scan 1 to scan 2 (ESV-2 - ESV-1) and NT PROBNP at 121-192 hours (r=0.41, p<0.05). Furthermore in the survivors there was a significant correlation between NT PROBNP levels at 121-192 hours and (ESV2-ESV1)/ESV1 (r=0.51, p=0.01). The correlation between NT PROBNP levels at 121-192 hours and (EDV2-EDV1)/EDV1 did not achieve significance (r=0.36, p=0.09).

Those patients with a poor outcome had higher NT PROBNP levels compared to the rest at 14-48 hours (931 $\pm$ 247 vs. 748 $\pm$ 170, p=0.01), 49-72 hours (731 $\pm$ 193 vs. 579 $\pm$ 138, p=0.006), 73-120h (559 $\pm$ 174 vs. 197

± 24.8, p<0.005) and 121-192 hours (1103 ± 387 vs. 823 ± 257, p=0.01) {Figures 8-11}.

There was no correlation of the diastolic dysfunction indexes, E/A reversal and E-deceleration times (DT) with NT PROBNP levels at any time following AMI.

DISCUSSION

This study is the first to report the optimum time for measurement of NT PROBNP following AMI. NT PROBNP measured at 73-120 hours following AMI was superior to measurements at other times in terms of identifying patients with significant left ventricular systolic dysfunction In both the short and medium term. Moreover, NT PROBNP predicted patients echocardiographic or poor clinical outcome over the 6 weeks following AMI.

Our study confirms that plasma levels of NT PROBNP are elevated in the early stages following AMI, reaching a peak within the first 48 hours following infarction before declining gradually over the ensuing 3 days. This is followed in a proportion of patients by a secondary rise in plasma NT PROBNP at around day 5, maintained 6 weeks later. This biphasic pattern of secretion of NT PROBNP was seen only in those suffering anterior infarction. This is analogous to the pattern of secretion observed for BNP following AMI [39]. This supports the supposition for an initial rise of NT PROBNP in response to acute tissue injuries in the early phase of acute myocardial infarction with a secondary rise possibly representing the ensuing development of infarct expansion and secondary evolution of LVSD.

Plasma levels of NT PROBNP were considerably higher following anterior compared to inferior site of AMI. In addition the biphasic pattern of secretion of NT PROBNP was not evident. This concords with the work of Morita who demonstrated that the biphasic rise of BNP-32 correlated with anterior site of infarction and the presence of LVSD [39]. These results suggest that not only the absolute value but also the pattern of NT PROBNP response may indicate the size of infarct and development of LVSD subsequent to AMI.

The mechanism(s) for prolonged elevation of NT PROBNP (at the OP visit) after AMI are not clear at the present time. Infarct expansion, defined as acute dilatation and thinning of the area of infarction not explained by additional myocyte necrosis in the subacute phase of AMI is observed more frequently in large anterior transmural infarctions [115]. Infarct expansion leads to increases in the size of the ventricular cavity which in turn increases the wall stress of infarcted and non-infarcted areas of myocardium resulting in ventricular remodelling. It is conceivable that increased wall stress secondary to infarct expansion with the subsequent development of ventricular remodelling stimulates the synthesis and secretion of NT PROBNP from ventricular myocytes.

We found a strong correlation between levels of NT PROBNP and WMI-1, the strongest correlation being with NT PROBNP measured between 73-120 hours following AMI. Importantly the correlation of NT PROBNP with WMI measured in hospital was observed despite wide variation in both age and acute management of patients. Furthermore this work has also demonstrated that measurement of NT PROBNP 4-5 days post AMI predicts ventricular function and outcome approximately 7 weeks later. This is in keeping with the study of Richards who demonstrated that NT PROBNP independently predicted LVEF at admission and 4 months

following AMI [53]. However the study of Richards looked at a single sample taken in the early post infarct period (between 2-4 days) [53]. Our study suggests that NT PROBNP measured after the acute phase (day 4-5) is a stronger predictor of poor clinical outcome as measured by LV systolic function and death. While NT PROBNP at all time points following AMI correlated strongly with WMI-1, plasma level at 73-120 hours was the only significant predictor of death or WMI-1 2 of $\leq$ 1.2. Moreover we have found NT PROBNP to be a predictor of the development of left ventricular enlargement and mitral valve regurgitation in the weeks following AMI. Thus it appears that NT PROBNP measured between 73-120 hours is most likely to provide prognostic information beyond 6 weeks.

Doppler derived indexes of diastolic dysfunction have been shown to be strong predictors of left ventricular remodelling and good predictors of prognosis following AMI [116]. However in our study we did not find any correlation between these Doppler derived indexes of diastolic function and NT PROBNP levels.

The concentrations of NT PROBNP recorded in our study, utilising an antibody against the C- terminal of NT PROBNP are considerably higher than the values determined in a previous study in which the antibody was directed against the N terminal of NT PROBNP [53]. Recently the N-terminal domains of preproBNP have been demonstrated to oligomerise through leucine 'zipper-like coiled-coil' motifs [90] (a short bundle of peptide α-helices that are wound into a superhelix) which function to promote the formation of peptide oligomers. It is possible that an antibody directed against N-terminal domains of NT PROBNP is potentially hindered from binding to its equivalent amino acid sequence. C-terminal domains however may be more readily accessible and detectable by immunoassay. Such differences in the differential immunoreactivities of the N- and C-

terminals of NT PROBNP are likely to account for the disparity between the concentrations of NT PROBNP observed between the two studies.

The antibody used in our work is specific for NT PROBNP, as demonstrated by the immunoreactivity of the antibody on SDS-Page Gels and the insignificant cross reactivity of the assay with ANP, BNP-32 and γ-BNP [89]. Additionally the chemiluminescent assay is a simple and inexpensive to perform and does not require the extensive safety measures of conventional radioimmunoassay. This furnishes the ease of clinical application of this assay.

The association between plasma concentrations of NT PROBNP and subsequent LVSD suggests measurement of this neurohormone would be appropriate in the routine clinical work up of patients in the immediacy of myocardial infarction. A blood test (easily taken alongside other biochemical markers) before discharge from hospital would predict underlying LV systolic function and therefore aid the individual stratification of patients. It may be argued that poor LV function is largely detectable clinically. We have previously shown NT PROBNP to be a stronger predictor of underlying LVSD than clinical examination [117]. Moreover in the current study, radiological heart failure was not predictive of LVWMI, unlike NT PROBNP. However until assays for NT PROBNP or alternative neurohormonal markers become routinely available, the use of a biochemical marker is unlikely to replace more established methods of assessment of ventricular function such as echocardiography.

In conclusion, NT PROBNP measured prior to hospital discharge following AMI predicts patients with significant impairment of left ventricular systolic dysfunction and at risk of poor outcome. Such measurements may help in identifying patients requiring appropriate pharmacological and

interventional therapy. The prospect of a new biochemical marker such as NT PROBNP for clinical assessment of LVSD is an exciting and challenging concept which may improve the therapy of post myocardial infarction patients.

TABLE 1:

Total number of patients	60
Male	45(75%)
Anterior site of infarction	39(65%)
Past Medical History	
AMI	4(6.6%)
Hypertension	16(26.6%)
Diabetes mellitus	4(6.6%)
Angina	10(16.6%)
Heart Failure on CCU	
Clinical LVF on CCU	24(40%)
Radiological evidence of LVF on CCU	35(58.3%)
Treatment on CCU	
Thrombolysis	52(86.6%)
Diuretic	25(41.6%)
ACE Inhibitor	16(26.6%)
Beta-blocker	33(55%)
Digoxin	2(3.3%)
Outcome	
Death (within 6 weeks)	4(6.6%)
LVSD (WMI < 1.2)	35(58.3%)
MR (grade 2 or 3)	14(23.3%)
Remodelling	5(8.3%)
Apical thrombus	5(8.3%)

TABLE 2:

	Mean (±SEM)
Biochemistry	
Plasma Sodium (mmol/l)	137.2 ± 0.41
Plasma Urea (mmol/l)	6.3 ± 0.24
Plasma Creatinine (mmol/l)	104.7 ± 2.9
Plasma Glucose (mmol/l)	9.3 ± 0.65
Peak CK (IU/L)	2153 ± 246
Echocardiographic indexes	
WMI-1	1.2 ± 0.06
EDV-1(ml)	106.9 ± 5.2
ESV-1(ml)	63.8 ± 4.0
WMI-2	1.5 ± 0.06
EDV-2(ml)	109.6 ± 6.5
ESV-2(ml)	64.3 ± 5.5

Figure 1:

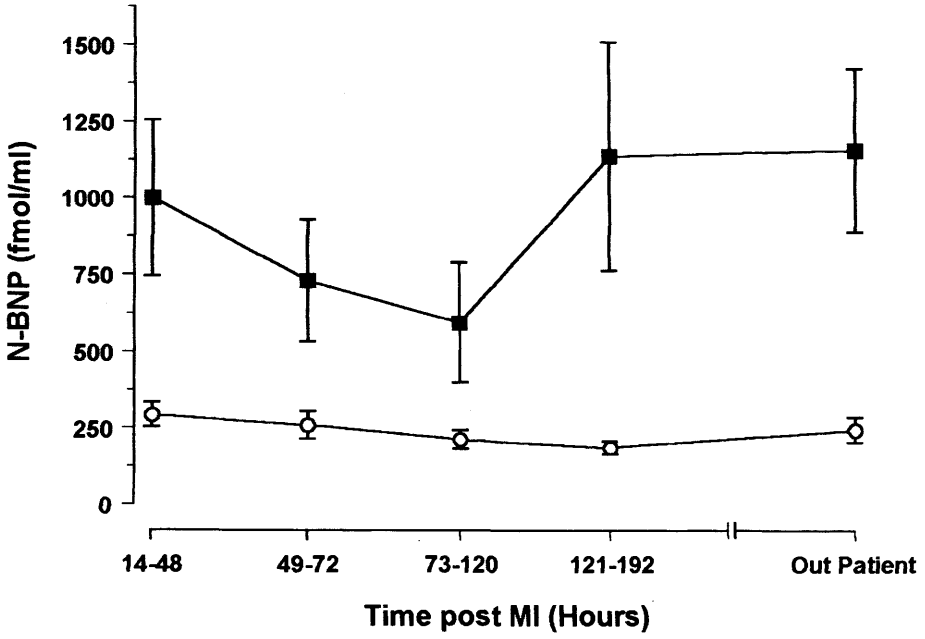

1500
1250
1000
750
500
250
0
N-BNP (fmol/ml)
14-48
49-72
73-120
121-192
Out Patient
Time post MI (Hours)

Figure 2: Correlation between Log NT proBNP at 14-48 hrs and WMI-1.

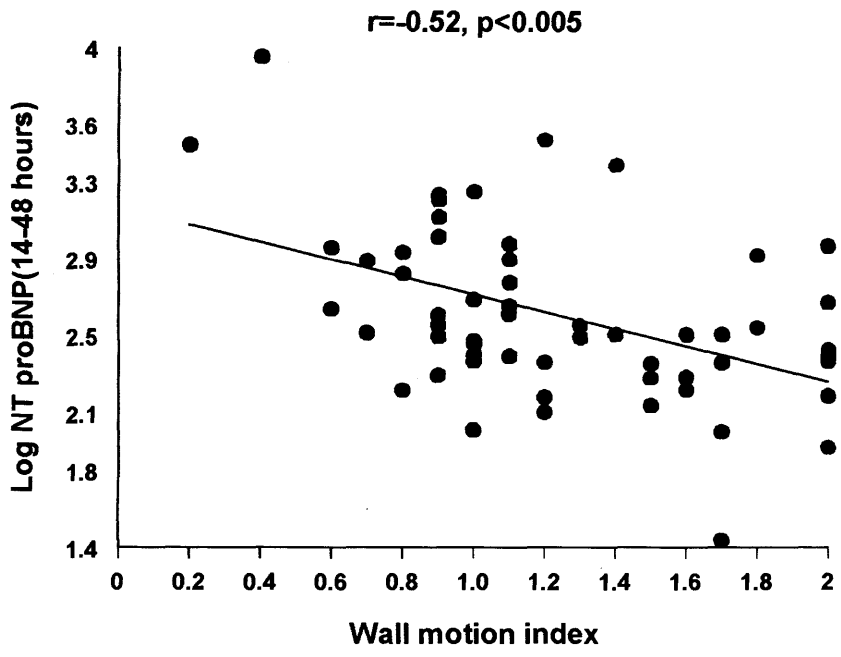


Figure 3: Correlation between Log NT proBNP at 49-72 hrs and WMI-1.

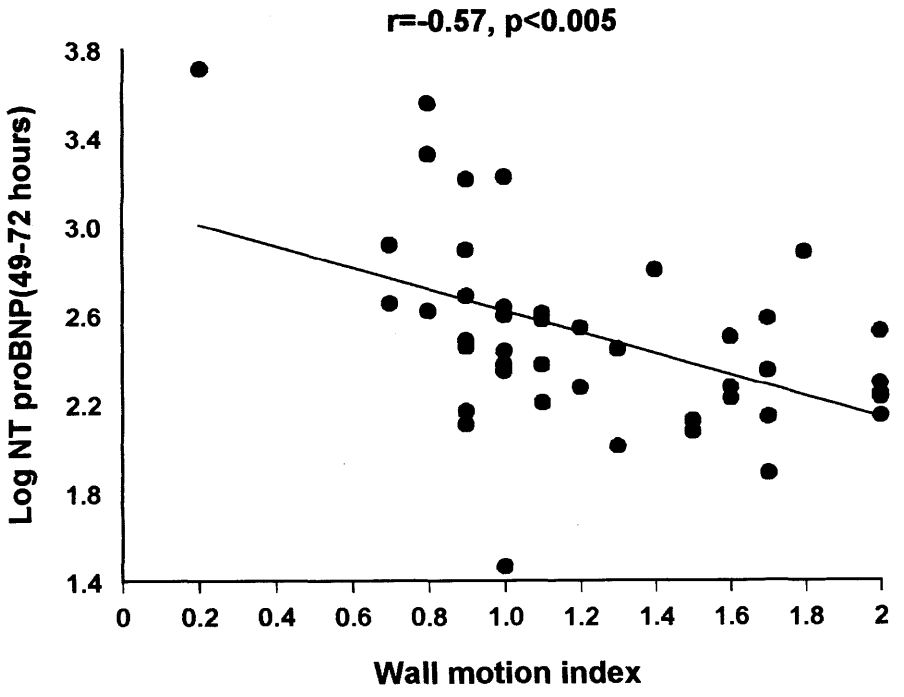


Figure 4: Correlation between Log NT proBNP at 73-120 hrs and WMI-1.

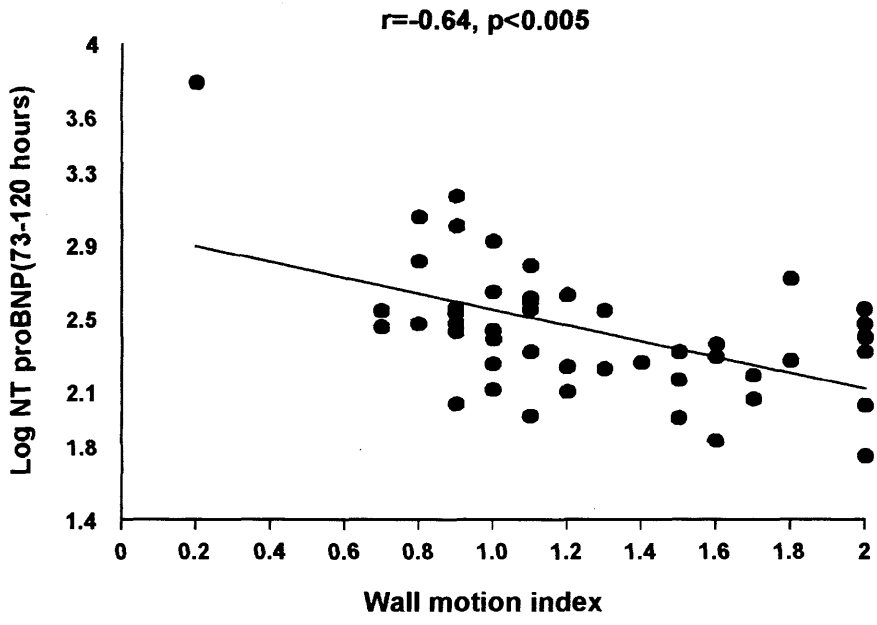


Figure 5: Correlation between Log NT proBNP at 121-192 hrs and WMI-1.

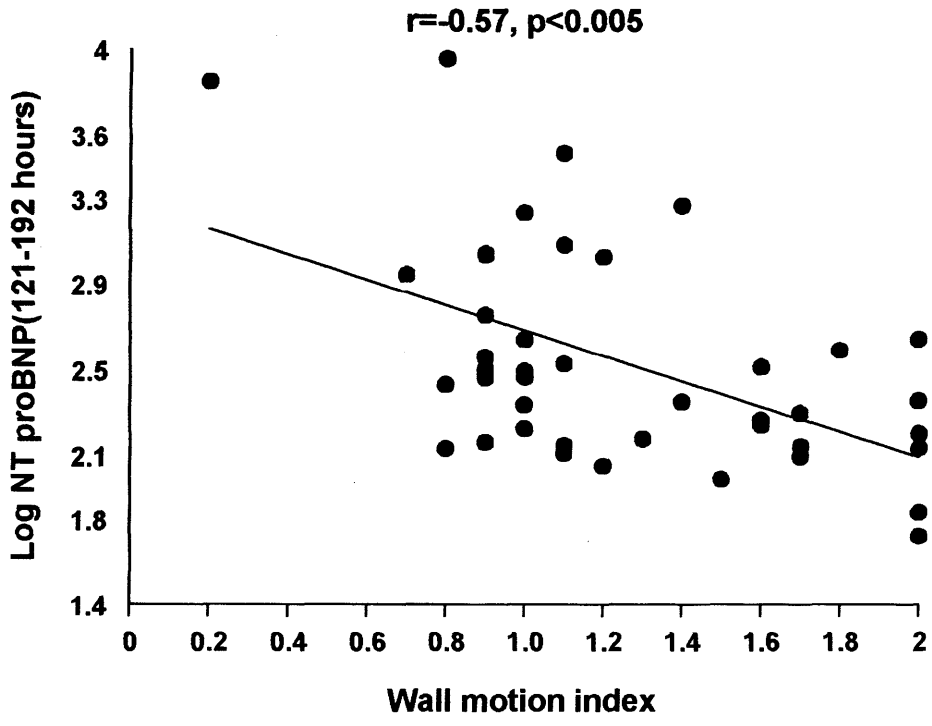


Figure 6: Correlation between Log NT proBNP at 14-48 hrs and WMI-2 in the survivors.

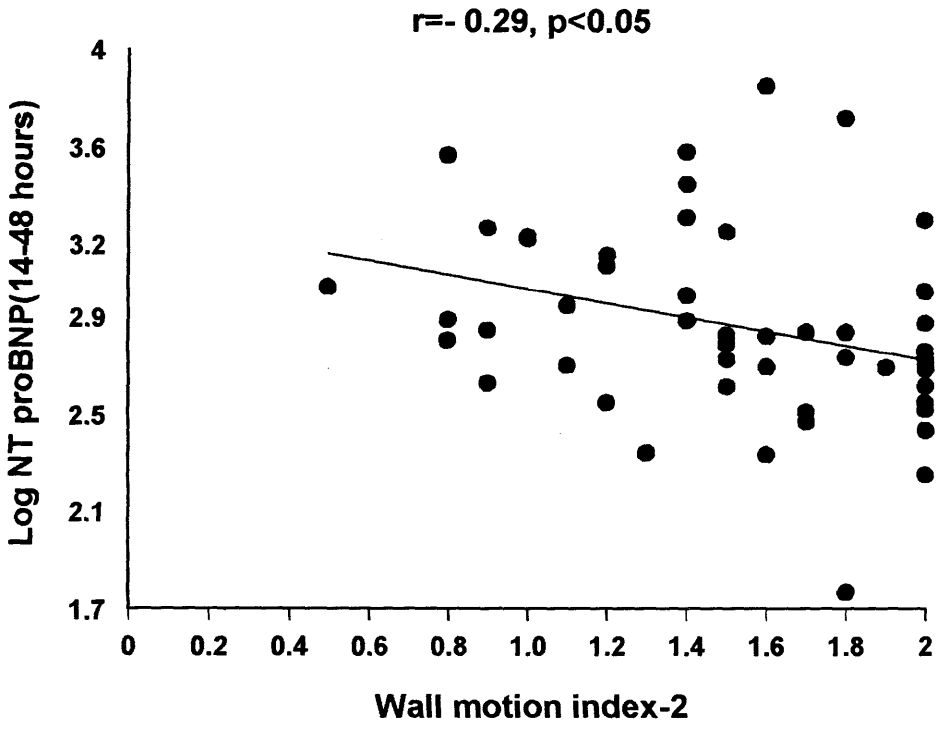


Figure 7: Correlation between Log NT proBNP at 73-120 hrs and WMI-2 in the survivors.

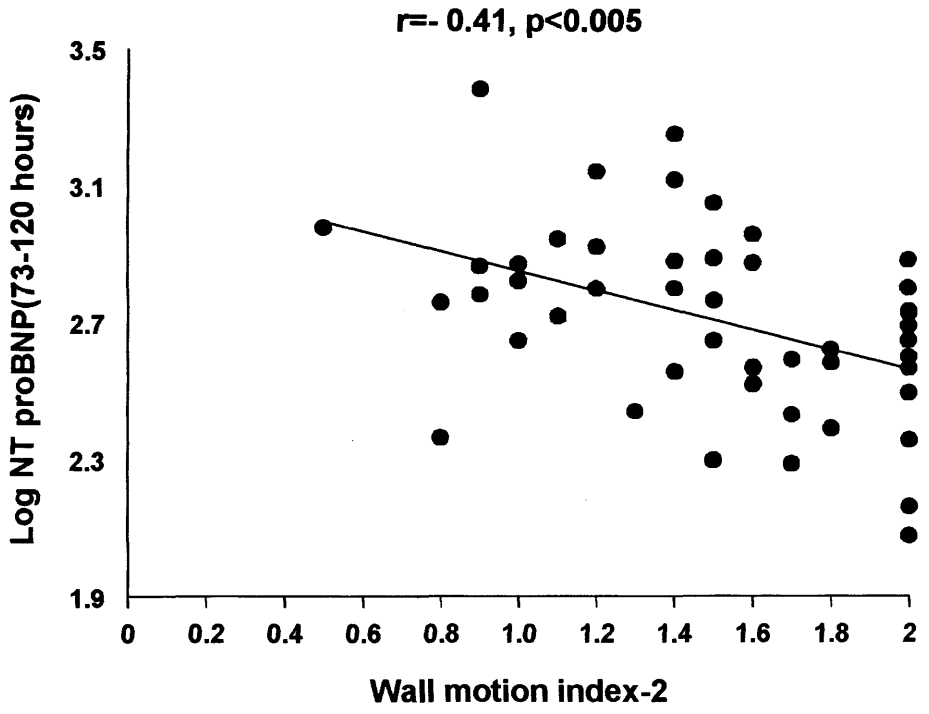


Figure 8: Box plot showing NT proBNP levels (between 14-48 hours) and those with a poor outcome vs. Those with a good outcome following the AMI.

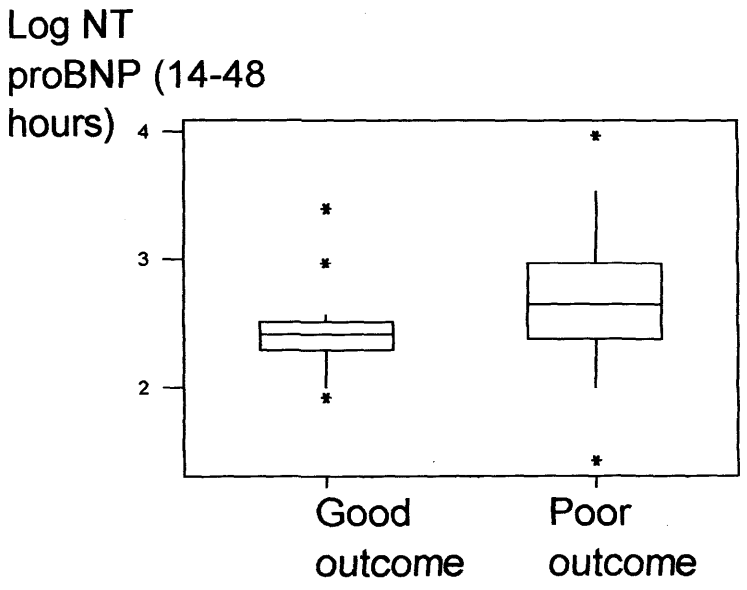


Figure 9: Box plot showing NT proBNP levels (between 49-72 hours) and those with a poor outcome vs. Those with a good outcome following the AMI.

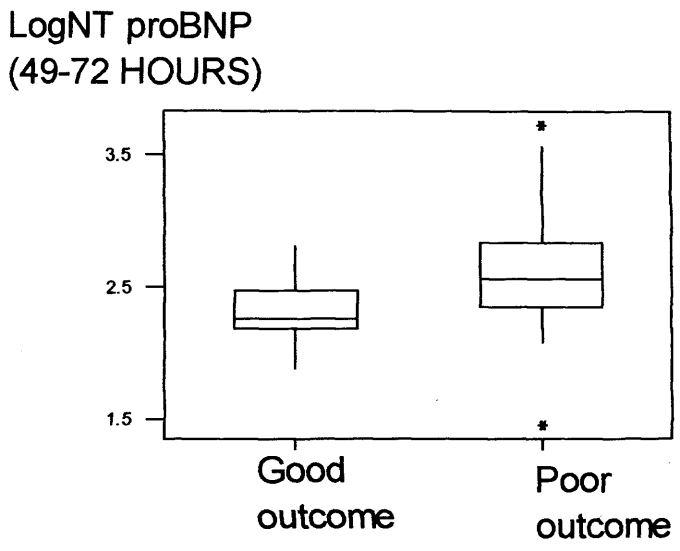


Figure 10: Box plot showing NT proBNP levels (between 73-120 hours) and those with a poor outcome vs. Those with a good outcome following the AMI.

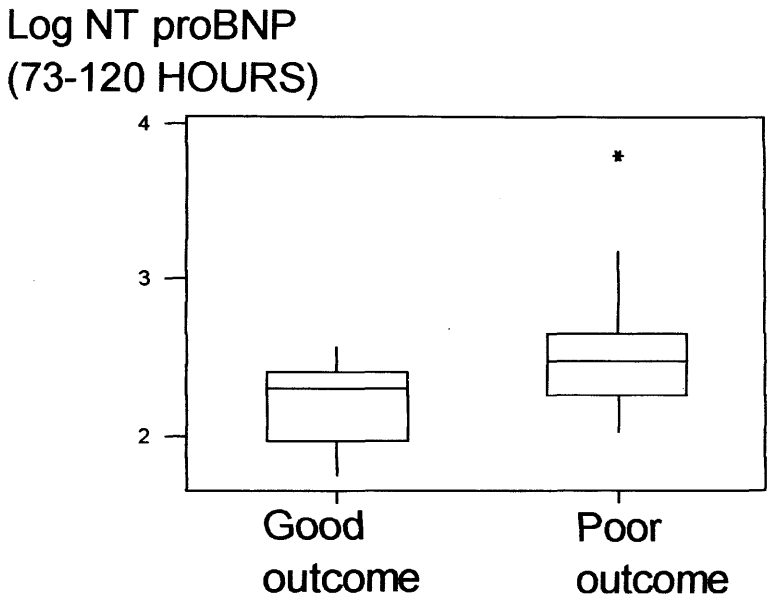
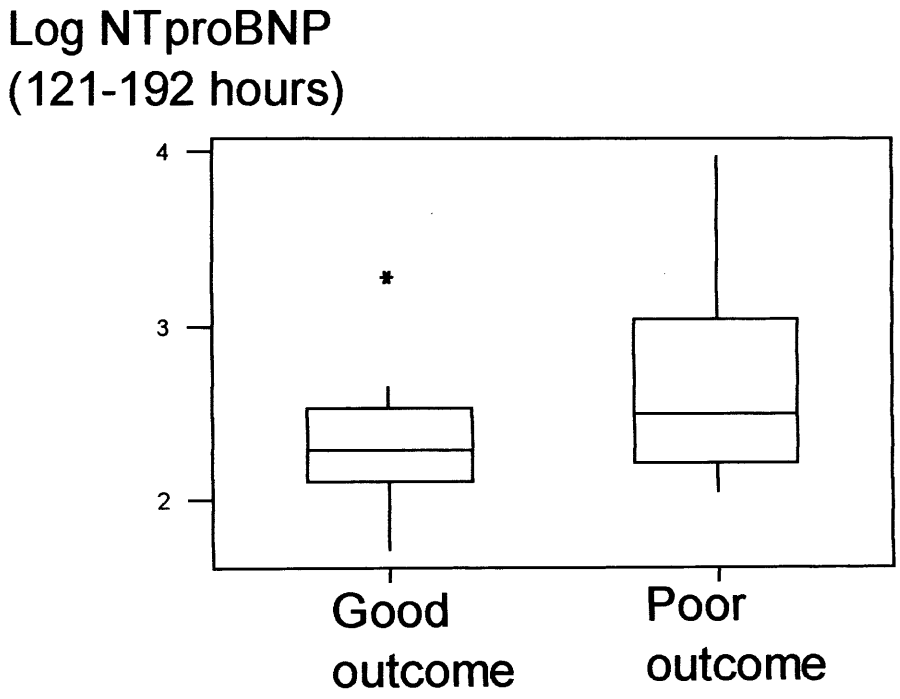


Figure 11: Box plot showing NT proBNP levels (between 121-192 hours) and those with a poor outcome vs. Those with a good outcome following the AMI.



CHAPTER 12

Plasma Cardiotrophin-1 Following An Acute Myocardial Infarction And Its Relationship To Left Ventricular Systolic Dysfunction.

INTRODUCTION :

More recently augmented gene expression of CT-1 and its receptor component have been shown to play a central role during ventricular remodeling after acute myocardial infarction (AMI) in a rat model [68]. Substantial overexpression of CT-1 and gp130 in the heart during experimental acute cardiomyopathy has been demonstrated [69]. Furthermore cardiomyocytes from patients with ischaemic cardiomyopathy are approximately 50% longer than normal, suggesting the addition of sarcomeric units in series [70,85].

These animal and morphometric experiments suggest a possible role for CT-1 in ventricular remodeling following AMI in man. There are no reports of the role of CT-1 in human physiology or pathology. The aim of this study was to examine circulating CT-1 levels in both the short and medium term following AMI in man and their relationship to left ventricular systolic dysfunction (LVSD).

METHODS

SUBJECTS

We studied 60 patients (45 Male, 39 anterior AMI, mean age 62.7 years, range 36-87) admitted to the Leicester Royal Infirmary Coronary Care Unit

with a diagnosis of Q-wave AMI. Serial measurements of plasma CT-1 were made at 14-48 hours, 49-72 hours, 73-120 hours, 121-192 hours in and at an out patient (OP) visit in survivors. In addition CT-1 was measured in 15 age matched normal controls (5 male, mean age 60.7 years, range 41-79).

Echocardiographic assessment of left ventricular function (see below) was assessed in 58 of the 60 patients during admission (WMI-1;Median day 4.5, range 2-6) and at the OP visit (WMI-2;Median day 50, range 20-73) in 52 of 56 survivors.

The study was approved by the local ethical and research committee and all subjects gave written informed consent to participation.

ECHOCARDIOGRAPHY

Left ventricular systolic function was assessed using was calculated using a nine-segment wall motion index (WMI) score and analysed by a single investigator (ST) blind to patient details and CT-1 results.

BLOOD SAMPLING

20 ml of venous blood was taken at each of 5 different time periods as above following the AMI and transferred into pre-chilled EDTA (1.5 mg/ml blood) tubes containing 500 IU/ml of aprotinin. Samples were immediately centrifuged at 4°C and the plasma separated and then stored at -70°C until assayed.

IMMUNOLUMINOMETRIC ASSAY FOR PLASMA CT-1

CT-1 levels were determined blind to patient details and echocardiographic findings. Each CT-1 value represents the mean of duplicate measurements.

STATISTICS

All statistical analyses were carried out using the software package Minitab (Minitab Inc, PA, USA). Concentrations of CT-1, age, plasma creatinine, plasma glucose on admission, peak creatine kinase (CK) and WMI scores were normalized by log transformation before analysis. All comparisons were by Student's t-test for unpaired data. Pearson's correlation coefficients were computed. Predictive models for the response variable (WMI) were developed using multiple linear regression analysis and stepwise logistic regression analysis. Comparisons with $p<0.05$ were considered significant. All results are expressed as means $\pm$ SEM in fmol/ml.

Results

Demographic and outcome data for the 60 patients are presented in Table 1, biochemical and echocardiographic data for the total population in Table 2.

CT-1 levels at 14-48h (108.1 $\pm$ 15.1), 49-72h (105.2 $\pm$ 19.7), 73-120h (91.2 $\pm$ 14.9), 121-192 h (118.8 $\pm$ 22.6) and at the OP visit (174.9 $\pm$ 30.9) were elevated when compared to control values (29.5 $\pm$ 3.6, $p<0.0001$). CT-1 levels were higher at the OP visit compared to all earlier time points ($p=0.01$, $p=0.008$, $p<0.0001$, $p=0.005$ respectively)(Figure 1).

At all time points mean CT-1 levels were higher in anterior compared to inferior AMI (136.9 ± 21.9 vs. 57.3 ± 8.1, p<0.005 at 14-48 h, 132.8 ± 28.6 vs. 51.9 ± 7.4, p<0.005 at 49-72 h, 109.7 ± 22.9 vs. 60.9 ± 9.4, p<0.05 at 73-120 h, 152.7 ± 32.1 vs. 50.9 ± 7.1, p<0.005 at 121-192 h and 233.4 ± 46.6 vs. 82.4 ± 16.4, p=0.006 at the OP visit). Mean WMI score in those with an anterior AMI was higher than in those with an inferior AMI (1.62 ± 0.07 vs 1.02 ± 0.06, p<0.0001).

In patients with anterior site of AMI, CT-1 levels were higher at the OP visit compared to all earlier times: 14-48 hours (p=0.05); 49-73 hours (p=0.02); 73-120 hours (p<0.005); and at 121-192 hours (p=0.05). In patients with inferior AMI, CT-1 levels at the OP visit was higher only when compared to that at 121-192 hours (p=0.03).

For the population as a whole, WMI-1 correlated with CT-1 at 14-48h (r= - 0.30, p<0.05), 49-72h (r= - 0.38, p<0.05, Figure 2), 73-120h (r= - 0.34, p<0.05) and 121-192 h (r= - 0.30, p<0.05). There was no significant correlation between CT-1 level at any time and WMI-2.

CT-1 levels at 14-48 hours correlated with those at 49-72 hours (r= 0.50, p<0.005), 73-120 hours (r= 0.53, p<0.005), 121-192 hours (r= 0.71, p<0.005) and CT-1 levels at the OP visit (r= 0.43, p<0.005).

We considered CT-1 at 14-48 hours, 49-73 hours, 73-120 hours, 121-192hours, age, gender, past history of angina, past history of AMI, history of hypertension, history of diabetes mellitus, plasma creatinine on admission, peak CK, clinical heart failure and radiological heart failure in multivariate models for the predictors of WMI-1. On best subsets analysis the strongest correlate with WMI-1 was CT-1 at 49-72 hours (R^2=20%,

$p<0.05$). The addition of a history of previous myocardial infarction improved diagnostic accuracy ($R^2=24\%$, $p<0.05$) of the model.

We considered the same variables in a multivariate model for the predictors of WMI-2. On best subsets analysis the strongest correlate with WMI-2 was past history of AMI ($R^2=12\%$, $P=0.007$). The addition of CT-1 at 14-48 hours improved diagnostic accuracy ($R^2=23\%$, $p<0.05$).

DISCUSSION

This study represents the first quantitative report of elevated plasma CT-1 in man following AMI. We have demonstrated sustained elevation of plasma CT-1 during the 7 days following AMI with a further increase in plasma levels at the OP visit. These changes were more prominent in patients who had suffered anterior site of infarction, a group of patients at high risk of LV remodeling and heart failure. We found a significant correlation between levels of CT-1 measured soon after AMI (strongest at 49-72 hours after AMI) and wall motion index assessed during admission. Furthermore early plasma CT-1 level was a strong independent predictor of LV systolic function.

The mechanism for initiation and maintenance of increased circulating plasma CT-1 following AMI are unclear. Hypoxia could be a potential stimulant for secretion of CT-1 in the acute phase. In addition, local myocyte stretch or infarct expansion may increase wall stress leading to increased synthesis and secretion of CT-1 [118]. In animal models CT-1 play a central role in promoting the process of left ventricular remodeling [68,69], generating a form of hypertrophy similar to the eccentric hypertrophy of volume overload [56]. In our study the highest levels of CT-1

were in patients with anterior infarction and in this group were highest at the OP visit. This continued secretion of CT-1 may suggest a possible role for CT-1 in the process of LV remodeling that contributes to the syndrome of heart failure after AMI.

While the physiological and therapeutic significance of our findings are as yet unclear, a number of cytokines (eg, interleukins, tumour necrosis factor alpha) are released after AMI which have pronounced local and systemic effects on the cardiovascular system [119]. These cytokines have been shown to be involved in the process of remodelling [83]. In this context it is of interest to note the stimulatory effect of CT-1 on BNP gene expression and peptide secretion [66, 67]. Plasma levels of BNP and its precursor pro-BNP are directly related to the degree of systolic dysfunction and to mortality following AMI [53]. It may be argued that elevated CT-1 level simply represents an earlier stage of the same neurohumoral cascade that results in elevated plasma BNP. However the relationship, if any, between CT-1 and BNP following AMI remains to be established. Moreover the identification of the earliest stages of such pathways may have important therapeutic implications in the future management of patients with heart failure. We may postulate that pharmacological manipulation of the CT-1 receptor may have therapeutic potential in the protection of cardiac cells from ischaemic injury. This would be the case particularly if the cytoprotective effects resulting from stimulation of the CT-1 receptor can be separated from the damaging eccentric hypertrophy inducing effects which result from continuous activation of the gp130 signaling pathway.

Our study is limited by the relatively small number of patients studied. This limits the ability of our study to quantify the relationship between plasma CT-1 and clinical outcome. Indeed the major limitation of this work is the inability to define the physiological significance or roles of increased CT-1

secretion in this population. There are as yet no pharmacological agents with which to manipulate the secretion or action of CT-1 in order to study its possible pathophysiological role in LV remodeling. Cardiac muscle survival is essential for the maintenance of normal cardiac function because adult cardiac muscle cells are terminally differentiated and have lost their proliferative capacity. However relatively little is known of the mechanisms required to maintain long term survival of cardiac myocytes. The acute secretion of CT-1 seen immediately following the AMI may have cytoprotective effects and as such confer an important role in cardiomyocyte salvage in the acute stages of AMI.

The relationship between CT-1 and known markers of adverse outcome following AMI, such as BNP, requires further investigation. Our study suggests the intriguing possibility that CT-1 may discriminate patients at risk of an adverse outcome. In this respect further studies with greater numbers of patients are required in order to fully assess the predictive value of CT-1. In addition, the potential cytoprotective effects of CT-1 in the early phases immediately following AMI warrant further investigation.

TABLE 1:

Patients	Number
Total	60
Male	45
Anterior site of infarction	39
Past Medical History	
AMI	4
Hypertension	16
Diabetes mellitus	4
Angina	10
Heart Failure on CCU	
Clinical LVF on CCU	24
Radiological evidence of LVF on CCU	35
Treatment on CCU	
Thrombolysis	52
Diuretic	25
ACE Inhibitor	16
Beta-blocker	33
Digoxin	2
Outcome	
Death (within 6 weeks)	4
LVSD (WMI $\leq$ 1.2)	35

TABLE 2:

	Mean (range)
Biochemistry	
Plasma Sodium (mmol/l)	137.2 (127-146)
Plasma Urea (mmol/l)	6.3 (2.9-11)
Plasma Creatinine (umol/l)	104.7 (46-177)
Plasma Glucose (mmol/l)	9.3 (5.7-30.9)
Peak CK (mmol/l)	2153 (487-8922)
Echocardiographic indexes	
WMI-1	1.2 (0.2-2)
WMI-2	1.5 (0.5-2)

Figure 1: Time course of CT-1 levels in those with anterior (■) and inferior (O) AMI.

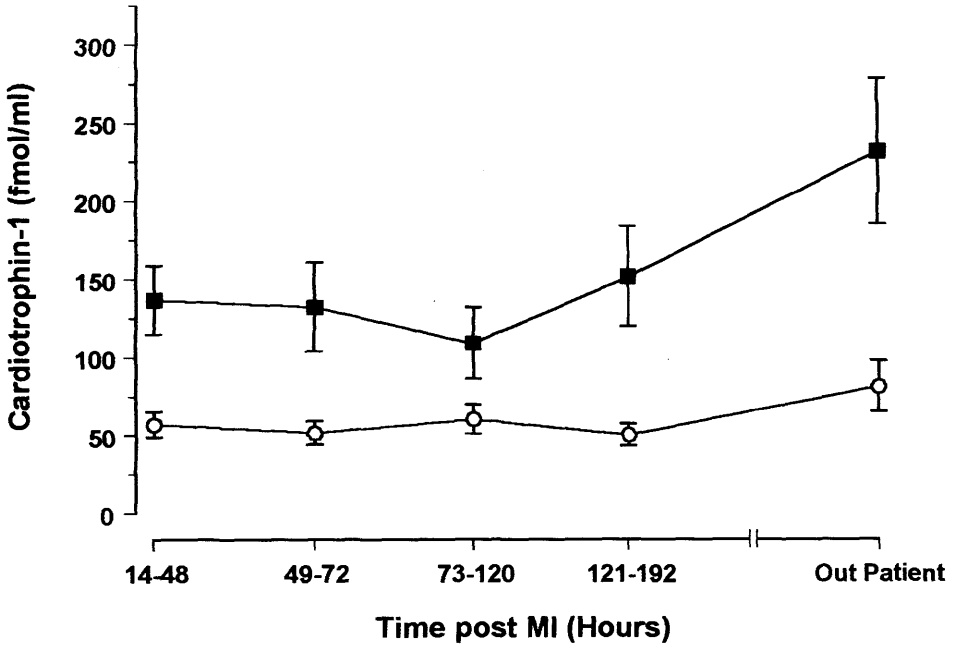


Figure 2: Correlation between Log CT-1 levels at 14-48 hours and Wall motion Index assessed during admission for index AMI.

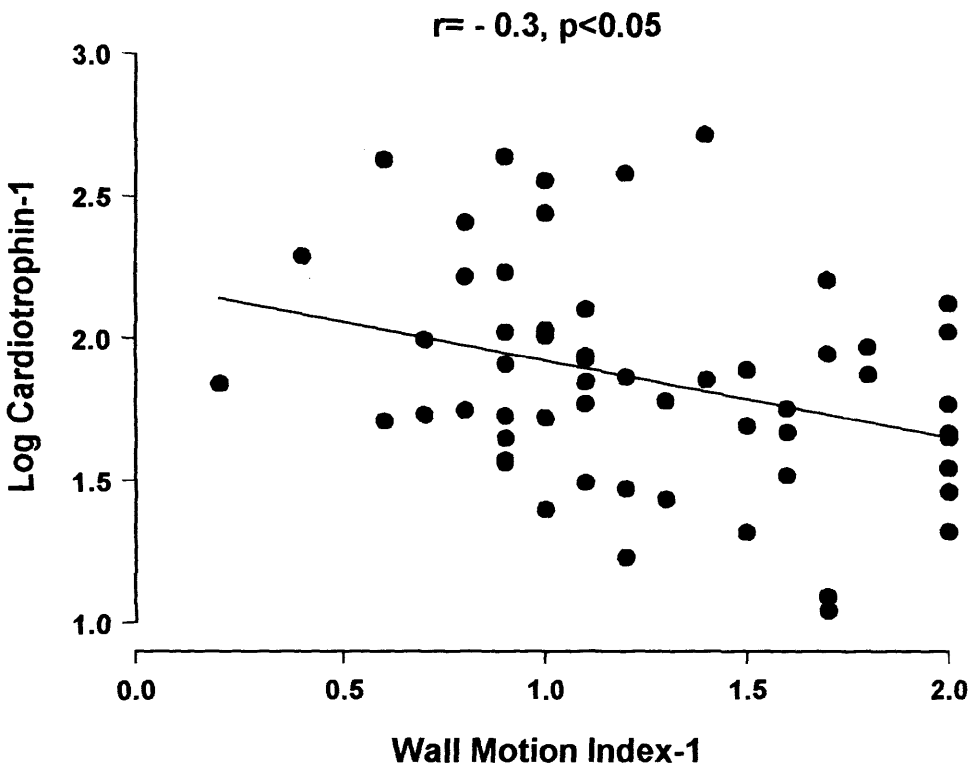


Figure 3 Correlation between Log CT-1 levels at 49-72 hours and Wall motion Index assessed during admission for index AMI.

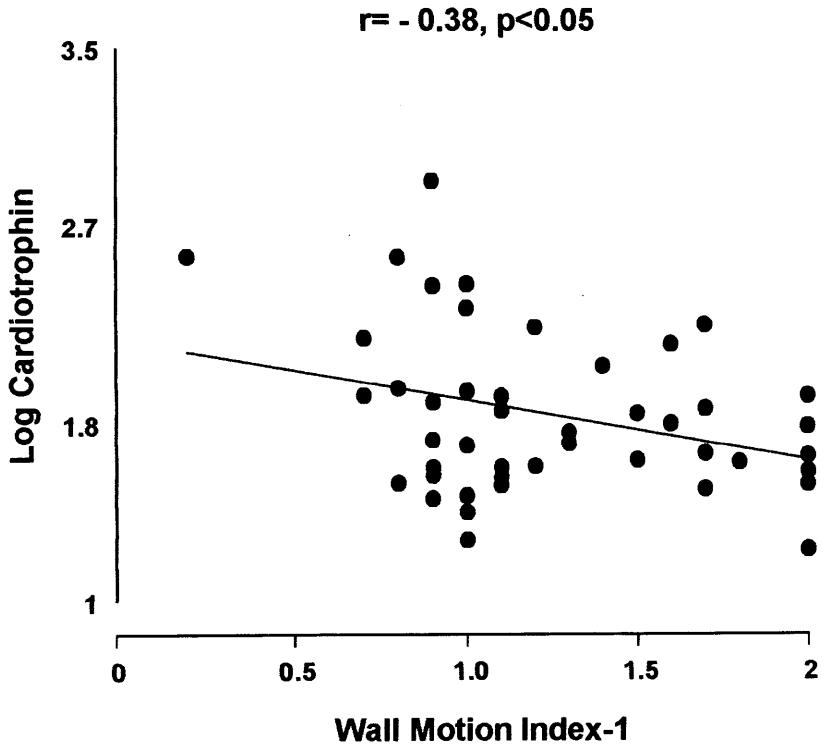


Figure 4 Correlation between Log CT-1 levels at 73-120 hours and Wall motion Index assessed during admission for index AMI.

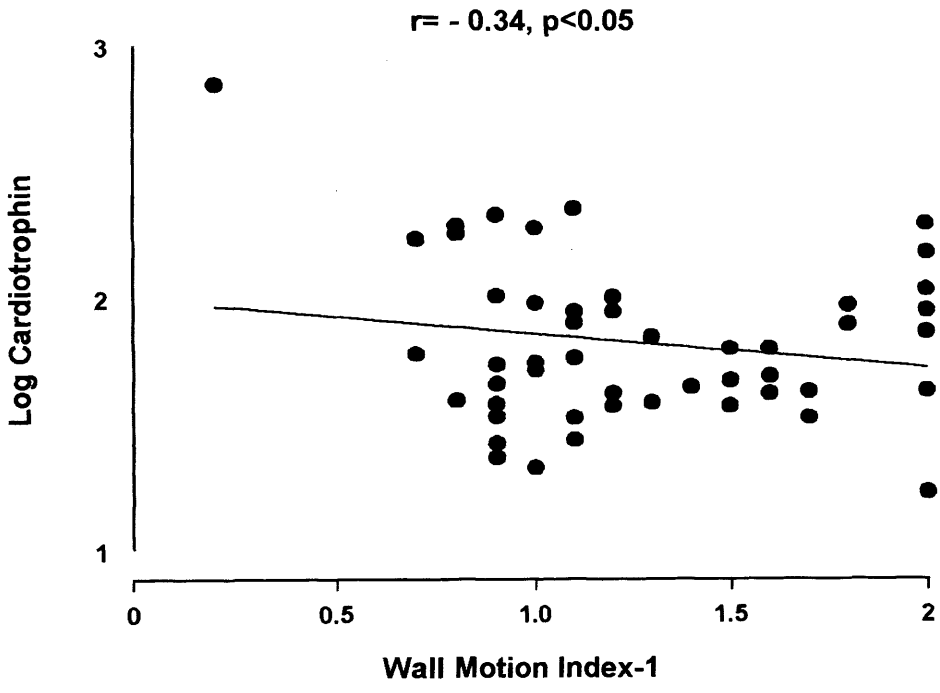
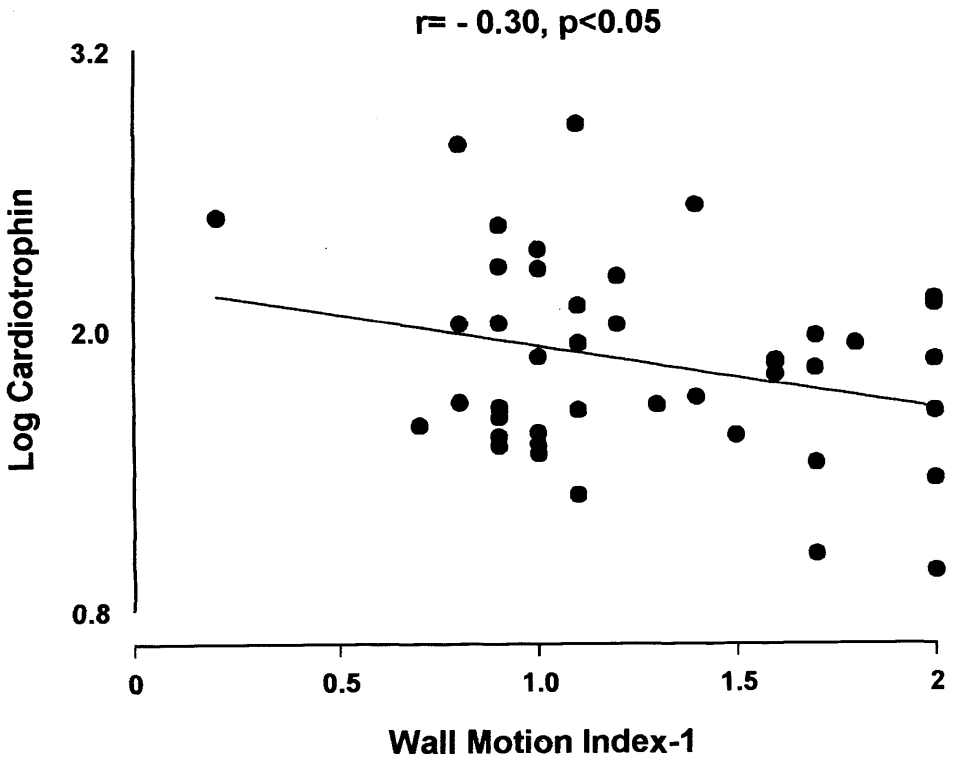


Figure 5 Correlation between Log CT-1 levels at 121-192 hours and Wall motion Index assessed during admission for index AMI.



CHAPTER 13

CONCLUDING REMARKS

One of the most promising areas of cardiological research at the present time is the study of cardiac natriuretic peptide secretion. BNP is a well known predictor of LV function and prognosis following AMI and in LVSD but NT-proBNP represents a potentially more discerning marker of these parameters. NT-proBNP has a similar pattern of secretion in patients with acute myocardial infarction as BNP and provides valuable information of left ventricular systolic function after AMI. As such routine measurement of plasma NT-proBNP would seem able to complement the routine investigation of patients with AMI. This would assist the individual stratification of patients, potentially avoid the discomfort of frequent blood sampling necessary for alternative enzymatic methods of determining infarct extent and ease the strain on established methods of determining functional impairment. Further evaluation is required to determine the value of measuring NT-proBNP as a means of population based detection for LVSD. However, the lack of complex handling and storage required and its ease of measurement from plasma are clearly beneficial attributes for a putative screening test.

More recently NT-ProBNP guided treatment of heart failure has been shown to reduce total cardiovascular events when compared with emperic therapy [120,121]. The benefit in monitoring our patients with heart failure using serial measurements to fine tune treatment in a more scientific way than we are able to do at present is extremely exciting. Neurohormonal analysis is rapidly moving from being a research tool to being a clinically useful test.

This study also represents the first quantitative measurement of Cardiotrophin-1 in humans. The classification of elements that determine the pathogenesis of remodelling and subsequent evolution of ventricular dysfunction remains a persistent effort of cardiovascular medicine. The potential action of cardiotrophin-1 on the pathophysiological process of LV remodelling could eventually lead to the development of targeted anti-cytokine therapy for heart failure with such an approach providing an additional facet to the pharmocotherapy of this condition.

The prospect of new biochemical markers such as NT-proBNP and Cardiotrophin-1 represents an exciting and challenging prospect as supplemental aids for the clinical assessment of various forms of cardiac disease and is probably just the dawn of a new era in cardiovascular medicine.

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