Processed BNP molecular forms as a new generation biomarker in cardiovascular disease

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Abstract: Processed BNP molecular forms as a new generation biomarker in cardiovascular disease

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B-type natriuretic peptide (BNP), a clinical biomarker for cardiovascular disease (CVD), is secreted by cardiac tissue in response to neurohormonal stresses. However, BNP is processed in circulation by peptidases to produce molecular forms. Clinical BNP measurements associate with prognosis in CVD, but associations with molecular forms are not known. This thesis investigated the prognostic role of three major circulating BNP molecular forms (5-32, 4-32 and 3-32) in patients with CVD.

BNP molecular forms were measured in plasma using an immuno-capture method followed by matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-ToF-MS) analysis. Circulating levels were investigated for associations with adverse events and compared to clinical measurements for prognosis and risk stratification.

BNP molecular forms independently predicted adverse events in patients with acute hospitalisations of heart failure (HF) (n=904) and myocardial infarction (MI) (n=1078), and predict death in chronic HF (n=1127). Prognostic ability was comparable with conventional BNP measurements for mortality from 6 months up to 3 years. Of the molecular forms, BNP 5-32 showed superior prediction qualities, indicating its utility in prognosis. Decision tree analysis showed the potential of BNP 5-32 as a secondary marker in MI after Global Registry of Acute Coronary Events (GRACE) scoring in identifying low-risk patients at 6 months. Serial measurements of BNP 5-32 showed that patients with elevated levels at follow-up were associated with worse outcomes.

The results presented in this thesis, for the first time, demonstrate that BNP molecular forms provide beneficial prognostic information for outcome in CVD patients in a comparative or superior manner to current clinical protocols. In the personalised medicine era, measurement of specific BNP forms may provide additive information in CVD and improve risk stratification. The niche for these molecular forms remains to be elucidated and further research is warranted for clinical translation.

List of publications, presentations and awards

Publications

Israr MZ, Heaney LM, Ng LL*, Suzuki T*. 2018. B-type natriuretic peptide molecular forms for risk stratification and prediction of outcome after acute myocardial infarction. *American Heart Journal*, 200:37-43.

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Contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACEi	angiotensin-converting enzyme inhibitors
ACTH	adrenocorticotrophic hormone
ACS	acute coronary syndrome
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
ARNI	angiotensin receptor neprilysin inhibitor
ATP	adenosine triphosphate
AUC	area under curve
BIOSTAT-CHF	a systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure
BNP	B-type Natriuretic Peptide
BP	blood pressure
BSA	bovine serum albumin
cGMP	cyclic guanosine monophosphate
CHAID	χ^2 automatic interaction detection (chi-square)
CI	confidence interval
CNP	C-type natriuretic peptide
COPD	chronic obstructive pulmonary disease
CVD	cardiovascular disease
DHB	2,5 dihydroxybenzoic acid
DPPIV	dipeptidyl peptidase 4
ECG	electrocardiogram
ECM	extracellular matrix

eGFR	estimated glomerular filtration rate
ESC	European Society of Cardiology
GRACE	Global Registry of Acute Coronary Events
GTP	guanosine triphosphate
HF	heart failure
HFrEF	heart failure with reduced ejection fraction
HFpEF	heart failure with preserved ejection fraction
HR	hazard ratio
IA	immunoassay
IHD	ischaemic heart disease
iPSC	induced pluripotent stem cells
JVP	jugular venous pressure
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LV	left ventricle
LVD	left ventricular dysfunction
LVEF	left ventricular ejection fraction
MACE	major adverse cardiovascular event
MALDI	Matrix-assisted Laser Desorption/Ionisation
MALDI-ToF-MS	Matrix-assisted Laser Desorption/Ionisation-Time of Flight-Mass Spectrometry
MI	Myocardial Infarction
MS	Mass Spectrometry
MS-IA	Mass Spectrometry Immunoassay

m/z	mass-to-charge ratio
NEP	neutral endopeptidase
NH_4HCO_3	ammonium bicarbonate
NP	natriuretic peptide
NPPB	Natriuretic Peptide Precursor B
NPR-A	Natriuretic Peptide Receptor A
NPR-B	Natriuretic Peptide Receptor B
NPR-C	Natriuretic Peptide Receptor C
N-STEMI	Non-ST-elevation myocardial infarction
HTN	hypertension
NT-proBNP	N-terminal pro B-type natriuretic peptide
NYHA	New York Heart Association
PCI	percutaneous coronary intervention
PBS	phosphate buffer saline
pg/mL	picogram per millilitre
PH	past history
QqQ	triple quadrupole
RAAS	Renin-Angiotensin-Aldosterone System
r_s	Spearman's rho
RSD	relative standard deviation
RVD	right ventricular dysfunction
SPSS	statistical package for the social sciences
Std β	standardised beta

STEMI	ST-Elevation Myocardial Infarction
TFA	Trifluoroacetic acid
ToF	Time of Flight
Troponin	cTn (Troponin I, cTnI; Troponin T, cTnT)
α-CHCA	α-cyano-4-hydroxycinnamic acid

CHAPTER ONE
INTRODUCTION

Parts of this chapter have been published elsewhere

Israr MZ*, Heaney LM*, Suzuki T. 2018. Proteomic biomarkers of heart failure. *Heart Failure Clinics*, 14:93-107

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1. INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is a term used to describe a group of diseases that involve the heart or blood vessels. Common types of CVD include chronic heart disease (CHD), peripheral arterial disease and cerebrovascular disease (stroke) which involve the blood vessels and; cardiomyopathy, heart failure (HF) and pulmonary heart disease which involve the heart.

CVD is the major cause of premature death in most European countries, whilst also being a major contributor of disability and economic burden on the health care system. It develops over many years usually caused by atherosclerosis and gives rise to more serious symptoms. CVD is a group of multifactorial diseases with risk factors such as hypertension, diabetes, smoking and high blood cholesterol, which tend to be related to diet and lifestyle, which have been shown to reduce mortality and morbidity when improved (De Backer et al., 2003).

The Global Burden of Disease study estimated that CVD was responsible for 29.6% of all deaths worldwide (15616.1 million deaths) in 2010 (Figure 1.1). This was double the number of deaths caused by cancers; more than all communicable, maternal, neonatal and nutritional disorders combined. The deaths for CVD are increasing due to a combination of ageing population, population growth and epidemiologic changes in CVD. In Europe, CVD is responsible for approximately half of all deaths, approximately 4 million deaths per year (Figure 1.1). This was greater in women (51%) compared to men (49%) (Nichols et al., 2014).

UK statistics for 2012 show that 28% of deaths were caused by CVD, only second to cancer (29%), showing a slight decrease in mortality and incidence. In women this was the largest cause of death, however for men cancer was responsible for more deaths. The main causes of CVD were CHD (46% of all CVD deaths) and stroke (26%) (Bhatnagar et al., 2015, Bhatnagar et al., 2016).

NHS England spent more than £6.8 billion treating patients with CVD in 2012/2013 with the greatest expenditure on secondary care (£4373 million), and within this the majority of the costs were due to emergency admissions. Primary care was responsible for 20.9% of the total expenditure on CVD with the majority of this spent on prescribing (£1387.5 million) (Bhatnagar et al., 2015). CVD is still an economic and health burden globally and nationally, despite declines in UK mortality rates.

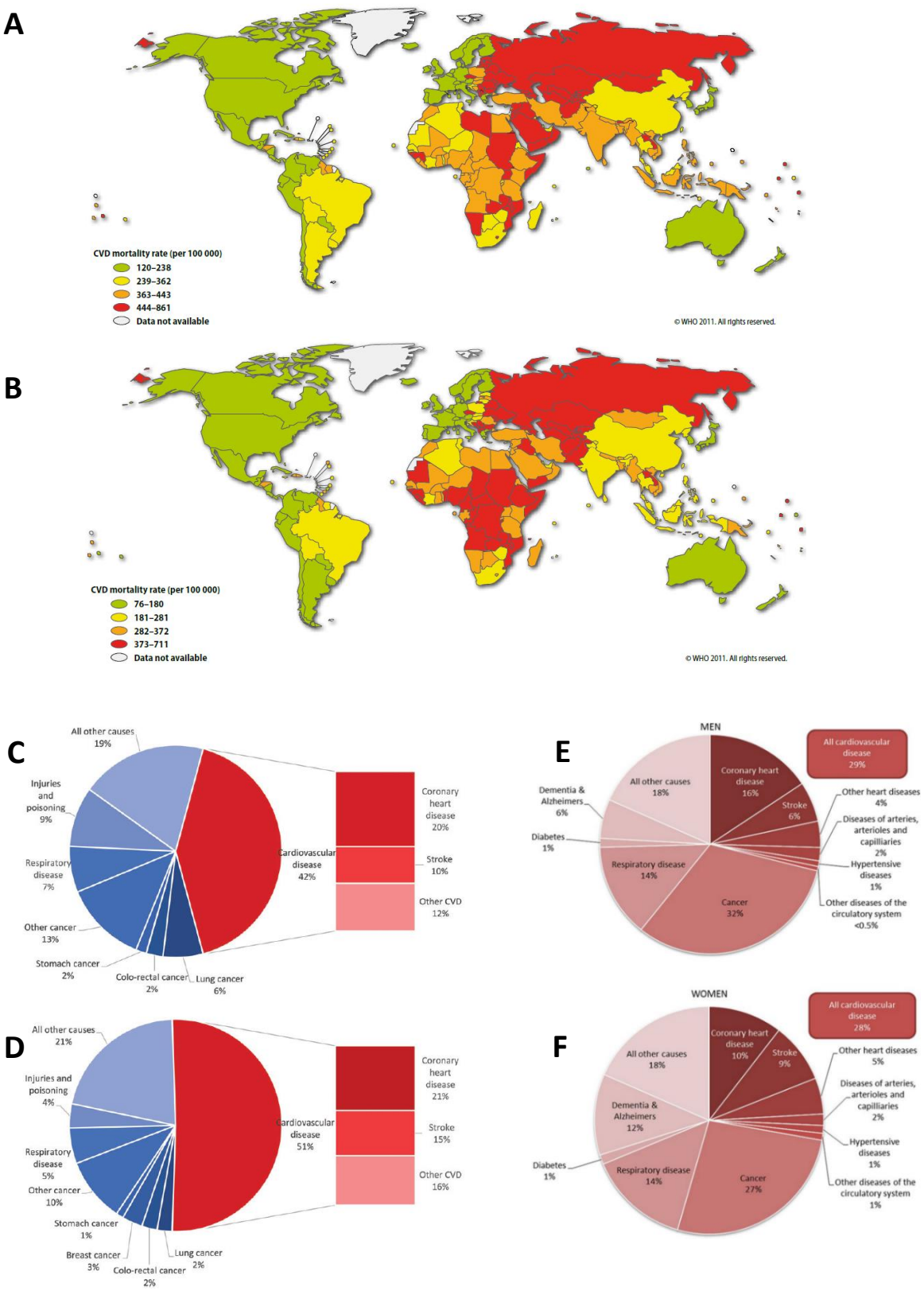


Figure 1.1 Epidemiological data for CVD. Global distribution for CVD mortality rates in (A) men and (B) women. Proportion of deaths due to major causes in Europe for 2013 in (C) men and (D) women. Proportion of deaths due to major causes in the UK for 2014 in (E) men and (F) women. (Mendis et al., 2011, Bhatnagar et al., 2015, Nichols et al., 2014).

1.2 Heart failure

Heart failure (HF) can be defined as a clinical syndrome caused by structural and/or functional cardiac abnormality resulting in reduced cardiac output. It is characterised by classical symptoms such as shortness of breath, swelling and fatigue alongside signs including peripheral oedema, and elevated jugular venous pressure (JVP) (Ponikowski et al., 2016).

1.2.1 Epidemiology and economic burden

HF is a major worldwide epidemic associated with high morbidity, mortality, and healthcare costs affecting more than 23 million, especially those aged ≥65 years (Roger, 2013). HF survival rates remain poor with 17-45% of admitted patients dying within one year and the majority dying within 5 years (Ponikowski et al., 2014).

1.2.2 Pathophysiology

The heart is a cone shaped, hollow, muscular organ located in the middle of the chest, between the lungs (middle mediastinum of the thorax) and the diaphragmatic dome. One-third of the heart on the right of the median plane and two-thirds to the left one. Its median weight is of about 250-300 grams, with a size that usually of one's own fist. The heart is divided into chambers, atria (right and left) and ventricles (right and left). Atria and ventricles are divided by two valves: tricuspid (among right chambers) and mitral (among left chambers). Two additional valves dived ventricles from major blood vessels originating from there (pulmonary and aortic, respectively from right and left ventricles) (Shaffer et al., 2014).

The right and left side of the heart are separated by the intraventricular septum which bulges slightly more to the right due to the thicker left ventricular wall. These chambers and valves are connected and supported by a fibrous skeleton surrounding the valves. The heart functions as a 'double-pump'. The 'pulmonary pump' with the right atrium and right ventricle operating as a low-pressure system pumping deoxygenated blood collected from the whole peripheral body to the lungs; the 'systemic pump' with the left atrium and left ventricle operating as a high-pressure system pumping oxygenated blood from the lungs to the body. Deoxygenated blood enters the right atrium via the

vena cava (superior and inferior) before passing to the right ventricle via the tricuspid valve and leaving this chamber via the pulmonary valve, through the pulmonary arteries, towards the lungs. Oxygenated blood from the lungs returns to the heart via the pulmonary veins into the left atrium before passing into the left ventricle via the mitral valve. The left ventricle has a much thicker wall compared to the right ventricle (10 mm and 3-5 mm respectively) allowing the left ventricle to pump the oxygenated blood to the body via the aortic valve and through the aorta (Mahadevan, 2018, Whitaker, 2014). Figure 1.2 depicts the basic anatomy of the heart showing the chambers and direction of blood flow.

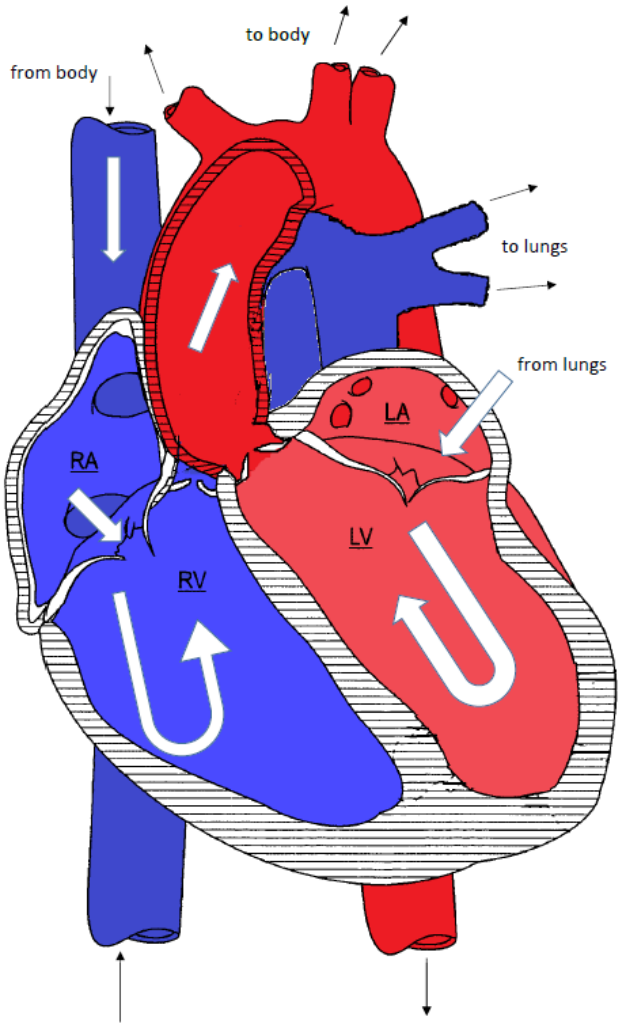


Figure 1.2 Basic anatomy of the heart showing the four chambers; RA, right atrium; RV, right ventricle; LA, left atrium; LV. Left ventricle. Also depicted is the direction of blood flow (arrows), the deoxygenated (blue) and oxygenated (red) parts of the heart. Image adapted from (Moaddeb et al., 2008)

The physiology of HF involves numerous factors and pathways such as cardiac stress and injury, neurohormonal activation and endothelial congestion, which can often worsen with disease progression (Mentz and O'Connor, 2016).

HF can be described as a condition resulting in the insufficiency of the heart to pump blood around the body to fulfil its needs. Normal cardiac physiology is therefore cardiac output or the amount of blood pumped out by the heart over a given time period. A product of heart rate and stroke volume, in a healthy heart this is 4-8 L/min. Factors affecting cardiac output include ventricular contraction and wall integrity whereas stroke volume can be affected by myocardial fibre stretch, resistance of the fibres in order for the ventricle to eject blood and contraction (Kemp and Conte, 2012).

HF begins following an event such as myocardial infarction (MI), heart disease, or myocarditis that produces reduced pumping capacity from the heart and the activation of compensatory mechanisms to counteract this. Compensatory mechanisms include the adrenergic nervous system, the renin-angiotensin-aldosterone system (RAAS) or the cytokine system which in the short-term are activated to stabilise the event. However in the long-term systemic and cellular changes occur, worsening the condition of the heart (Schwinger, 2010).

Numerous proposed models or compensatory mechanisms have been outlined as contributing pathophysiology of HF; haemodynamic model, extracellular matrix model, cardiorenal model, neurohumoral model, abnormal Ca^{2+} cycling model, cell death model and genetic model (Braunwald, 2013).

Left ventricular dysfunction

Left ventricular dysfunction (LVD) is a common cause of HF and perhaps a final pathway for a number of other cardiac disorders occurring due to mechanical changes in cardiac performance. LVD can involve the whole of the left ventricle or part of it and be either systolic or diastolic (Iliceto, 1997). Compensatory mechanisms are triggered after continuous effects of LVD which result in cardiac changes. These changes involve both ventricular remodelling and neurohormonal changes leading to the ventricles thickening and increasing in size or dilating without necrosis due to ventricular expansion (Armstrong, 2000). Systolic dysfunction is result of the left ventricle

weakening and often increasing in size. Stroke volume and cardiac output are reduced and the ventricles are unable to pump blood efficiently enough to meet the demands of the body triggering a series of compensatory mechanisms (Serdahl, 2008). Diastolic dysfunction results in the ventricle unable to relax whilst filling. Abnormalities include impaired filling and delayed relaxation which could be due to increased mass, changes in collagen network responsible for myocardial elasticity or factors promoting fluid retention. Diastolic dysfunction is less common that systolic (Gaasch and Zile, 2004).

Right ventricular dysfunction

Right ventricular dysfunction (RVD) can arise either primarily due to disease of the right ventricle or secondary to LVD in HF. The latter may occur after an increase in pulmonary venous and arterial pressure due to an increase in LV failure after afterload. This could simultaneously take place in both the right and left ventricles along with myocardial ischaemia. LVD could result in systolic driving pressure decreasing in the right ventricular coronary perfusion, resulting in septal dysfunction and left ventricular dilation in a limited area may limit RVD. Alternatively pressure overload in the right ventricle may lead to left ventricular failure (Voelkel et al., 2006).

Haemodynamic model

The haemodynamic or cardiocirculatory model is a model where HF occurs due to abnormalities in the pumping capacity of the heart and peripheral vasoconstriction. This model was the basis for the use of vasodilators to increase cardiac output (E Louridas and G Lourida, 2012). Haemodynamic changes occur from ventricular remodelling and changes with the type of HF. In HF with reduced ejection fraction (HFrEF) the left ventricular chamber is dilated and the left ventricle mass:end-diastolic volume ratio is reduced or normal. However, in HF with preserved ejection fraction (HFpEF) the left ventricular chamber is normal, the wall is thicker and the left ventricle mass: end-diastolic volume ratio is increased (Braunwald, 2013).

Extracellular matrix model

The extracellular matrix (ECM) is an important component of the heart. The structure and function of the heart can therefore be affected by factors affecting the integrity of

the ECM. Following MI, necrosis of the myocardium is triggered leading to its myocardium. This causes a release of growth factors in the connective tissue forming fibroblasts which are involved in tissue repair (Fan et al., 2012).

The process of remodelling alters the structure and function of the ECM which can affect the repair and remodelling process of the myocardium. Factors that affect the ECM in cardiac remodelling include; amount of ECM, composition and geometry of matrix proteins such as collagen and elastin contributing to the stiffness or elasticity (Miner and Miller). Inadequate synthesis can results in ventricular wall thinning, aneurysms, and impaired pump function such as decreased or delayed relaxation. Increased synthesis results in increased stiffness and slow or incomplete filling of the ventricles (Katz and Zile, 2006).

Cardiorenal model

This model is based on the connection between the heart and kidneys on sodium and water retention, central to the role of two common manifestations of HF; oedema and dyspnoea. Commonly diuretics and dietary sodium restriction are used to manage congestion in HF. In severe HF, when this type of therapy is intensified this can lead to renal failure (Braunwald, 2013).

Neurohumoral model

Neurohumoral or neurohormonal model is based on the effects of neurohormones such as noradrenaline, angiotensin II, tumour necrosis factor (TNF) and natriuretic peptides. Identified in the 1960s, it was shown that the activation of the adrenergic nervous system in healthy subjects increased myocardial contractility and redistribution of cardiac output during exertion. The most powerful compensatory mechanism aimed to support the heart is suggested to be the adrenergic nervous system. The function of this mechanism and indeed other neurohumoral mechanisms initiate a vicious cycle. In HF, activation of the adrenergic system or the RAAS results is increased contractility of the myocardium, which in turn stimulates the depressed contractility of the heart causing vasoconstriction and increased blood pressure. However, if the activation of these systems is prolonged, further damage to the myocardium can occur by maladaptive

remodelling of the ventricles. It is by these mechanisms that two common therapies, angiotensin-converting enzyme (ACE) inhibitors and β-adrenergic blocking agents are made to function (Braunwald, 2013, Mann and Bristow, 2005).

Abnormal Ca^{2+} cycling model

In the healthy heart Ca^{2+} regulates cardiac muscle contraction after being released by the sarcoplasmic reticulum (SR) which is dependent on Ca^{2+} cycling kinetics regulated by phosphorylation and dephosphorylation of proteins involved in muscle contraction. In HF, defects in the SR Ca^{2+} handling is characterised by leaky ryanodine receptor 2 (RyR2) which results in diastolic SR Ca^{2+} leak, reduced SR Ca^{2+} content and a decreased Ca^{2+} for short-term. Impaired SR Ca^{2+} uptake can add to this problem due to reduced activity of the SR Ca^{2+} (SERCA2a) uptake pump which occurs due to reduced expression or inhibition of the pump (Marks, 2013). The involvement of defective SERCA2a and RyR2 can result in impaired cardiac contractility and therefore HF.

Cell death model

Myocyte apoptosis is an important mechanism in HF and occurs from developmental defects in blood flow in an overloaded heart resulting in fibrosis in the ventricular wall (Olivetti et al., 1997). In contrast to apoptosis, necrosis and autophagy-associated cell death are also shown to occur in failing hearts, with autophagy-associated cell death suggested to be the most common form (Konstantinidis et al., 2012). Increased rate of cell death is dependent on many factors including; elevated neurohormone levels, excessive adrenergic activity, inflammation, oxidative stress, toxins such as alcohol or chemotherapeutic drugs, and age (Braunwald, 2013).

Genetic causes

The earliest clinical event showing a genetic cause of HF is ventricular remodelling where signs of left ventricular hypertrophy or dilation may occur. Genes that function to initiate contraction have shown mutations such as sarcomere protein mutations, dystrophin-associated glycoprotein mutations and Z-disc protein mutations. Mutations involving energy generation and regulation allowing contraction to occur fully include mitochondrial mutations and nuclear-encoded metabolic mutations. Also mutations

affecting Ca^{2+} cycling which regulates cardiac muscle contraction include RyR2 mutations and ATP-sensitive potassium channel mutations (Morita et al., 2005).

1.2.3 Aetiology

HF is a multifactorial disease with a range of causes both heart-related and unrelated. The most common cause of HF leading to functional deterioration are damage or loss of heart muscle, ischaemia, increased vascular resistance, hypertension and tachyarrhythmia. CHD is the most common cause of HF being responsible for approximately 70% of HF causes. Other causes include drugs, toxins, cardiomyopathies, endocrine and nutrition (Dickstein et al., 2008, Fox et al., 2001).

Generally in elderly individuals, HF does not occur alone and is often caused by an underlying cardiac defect in combination with other medical conditions, which quite often the patient is being treated for. Cardiac problems which cause HF include angina, arrhythmias and hypertension. Other non-cardiac contributing causes include osteoarthritis which is an age-related cause, gout which is the result of diuretic treatment, renal failure which is a common comorbidity with many causes, as well as many unexplained causes including anaemia, depression and cachexia (McMurray and Pfeffer, Braunstein et al., 2003).

Factors contributing to HF hospitalisation identified in most patients included respiratory conditions (15.3%), ischaemia (14.7%), arrhythmia (13.5%) and uncontrolled hypertension (10.7%) (Fonarow et al., 2008a).

1.2.4 Classification

There are many ways of classifying the severity of HF and can be based on functional classification or severity of symptoms. The New York Heart Association (NYHA) classification system is a commonly used system based on an estimate of a patient's functional ability based on symptoms (Table 1.1). Generally, therapies for HF are administered to functional class III and IV, but not class I and II. However, the definitions of these classes makes it easy to differentiate between class I and IV patients with the severity of symptoms correlate poorly with measures of left ventricular function (Russell et al., 2009).

The Killip classification can also be used for the severity of the patient's condition and HF severity in acute myocardial infarction (MI) (Table 1.2).

Table 1.1 New York Heart Association (NYHA) classification system for heart failure based on patients functional capacity. Adapted from (Hurst et al., 1999).

NYHA Class	Definition
Class I	Cardiac disease but with no symptoms and/or limitations experienced during physical activity. No symptoms from ordinary activities such as fatigue, palpitations, dyspnoea and angina.
Class II	Cardiac disease with mild symptoms and/or limitations during physical activity, whilst patients experience comfort at rest. Mild symptoms include fatigue, palpitations, dyspnoea and angina.
Class III	Marked limitations during any activity, whilst patients experience comfort at rest. Any type of activity causes fatigue, palpitations, dyspnoea and angina.
Class IV	Cardiac disease resulting in inability of the patient to carry out any type of physical activity without experiencing difficulty. Symptoms of heart failure are even present at rest.

Table 1.2 Killip classification system for heart failure based on the severity of the patient's condition. Adapted from (Mello et al., 2014).

Killip Class	Definition
Class I	No clinical signs of heart failure
Class II	Rales or crackle in the lungs, third heart sound (S_3) and elevated jugular venous pressure
Class III	With acute pulmonary oedema
Class IV	With cardiogenic shock or arterial hypotension and peripheral vasoconstriction

1.2.5 Acute and chronic heart failure

Acute HF can be defined as a rapid change in HF signs and symptoms and therefore requires emergency attention and treatment. Chronic HF describes patients who have had HF for some time, and those who have been treated and experience unchanged signs and symptoms for at least 1 month are said to be 'stable'. Symptoms in acute HF result from left ventricular filling pressure and patients often have other conditions including coronary heart disease, hypertension and/or renal disease which contribute to the effects of acute HF. Acute HF could be present as *de novo* HF or be due to worsening acute decompensation chronic HF, or advanced HF with severe left ventricular systolic dysfunction (Ponikowski et al., 2016). Management of acute HF involves improving haemodynamic status, relieving pulmonary congestion and improving tissue oxygenation (Millane et al., 2000).

1.2.6 Diagnosis

HF is occasionally the final stage of heart disease and can be difficult to diagnose due its multifactorial, multi-organ presentation and the obscurity of signs and symptoms during early stages of the disease (Bui et al., 2011).

Diagnosis typically begins with signs and symptoms.

1.2.6.1 Signs and Symptoms

Many of the most common signs and symptoms of HF may be difficult to interpret especially in the elderly, obese and in women (Swedberg et al., 2005).

HF symptoms vary from one individual to another however, the most common symptoms are shortness of breath, fatigue and swollen ankles and legs (oedema). Other symptoms include lung crepitations, pulmonary oedema, dyspnoea, coughing, hepatomegaly and pleural effusion. Clinical suspicion is therefore confirmed by testing cardiac function (Paulus et al., 2007).

These common signs and symptoms are not specific to HF and as individual symptoms they are weak predictors and show poor reliability. A systematic review showed that the more reliable and specific symptoms include (% specificity); history of myocardial infarction (MI) (89%), orthopnoea (89%), cardiomegaly (85%), heart sounds (99%), lung

crepitations (81%) and hepatomegaly (97%). In terms of sensitivity, orthopnoea showed the best sensitivity (87%) to rule out HF in primary care. However, since the majority of patients with HF present with this symptom, sensitivity of 87% is not enough to rule out HF based on this symptom alone (Hobbs et al., 2010).

1.2.6.2 Diagnostic tests

Patients with suspected HF are referred for clinical testing. Although there is no gold standard, NICE recommend transthoracic Doppler 2D echocardiography and measurement of serum natriuretic peptides (NP) (see section 1.5) to rule-out HF.

Other tests may include;

- Electrocardiogram (ECG), recommended by the ESC to be performed on every suspected case of HF. Although highly sensitive (89%), the specificity is low-to-moderate and should not be used to rule out HF (Dickstein et al., 2008, Mant et al., 2009). A widely available, rapid and non-invasive technique providing information about the electrical activity and wall structure of the heart.
- Echocardiography, ultrasound imaging techniques including transthoracic Doppler 2D. Echocardiography is a widely available and non-invasive for HF that was described by ACC/AHA as 'the single most useful diagnostic test in the evaluation of patients with HF.' Echocardiography provides information for the size and shape of the heart, evidence of heart damage, pumping capacity, haemodynamic status and left ventricular ejection fraction. Sensitivity and specificity differs for different techniques however they are reported to have similar results to NPs (Dokainish et al., 2004, Kirkpatrick et al., 2007).
- Chest X-ray, one of the first diagnostic tests following patient history and signs and symptoms. Chest X-ray sensitivity for HF were reported to be approximately 68% whereas specificity ranged from 76-83%. Chest X-rays provide evidence of suspected HF such as pulmonary congestion and cardiomegaly, however they do not perform well as independent predictors of HF (Hobbs et al., 2010).
- Blood tests (electrolytes, urea, creatinine, eGFR, thyroid function tests, liver function tests, glucose, lipid profile, and full blood count), urinanalysis and spirometry.

1.2.7 Prognosis

Prognosis of HF is poor and worsens with age. It is associated with a poor quality of life and a high rate of mortality. HF impacts heavily on the quality of life showing that the physical health burden in heart failure was greater than that suffered in other common chronic disease states (Hobbs et al., 2002). HF is multifactorial and the worsening effects on the quality of life and therapies are available to control the symptoms, however even still HF worsens over time and is still associated with a 10% mortality rate (Neubauer, 2007). The Framingham Heart Study showed survival rates were better in women compared to men whilst mortality rate increased with age. The 1 year survival in women was 64% compared to 57% in men and the 5 year survival rate was 38% in women compared to 25% in men (Ho et al., 2003). A study comparing first admission HF patients with cancer patients showed the 5-year survival rate for HF in 1991 was 25%, lower than many common types of cancer. First admission for HF commonly shows worse survival rates compared to myocardial infarction and most types of cancers, with the exception of lung cancer. The number of deaths in males for HF were similar to those with lung cancer, and in women HF deaths was greater than deaths due to cancer (Stewart et al., 2001).

Despite this, results have shown that HF incidence has been stable over the past 20 years with survival rates increasing overall, however women and the elderly populations have shown less improvement (Roger et al., 2004). Efforts to improve HF prognosis and management of HF rely on therapies, lifestyle changes and palliative care.

1.2.8 Treatment

The aim of treatment in HF patients is to improve their clinical status, quality of life, functional capacity whilst preventing hospital admissions and reducing mortality. Frequently prescribed treatments in all symptomatic patients with HFrEF are angiotensin-converting enzyme inhibitors (ACEi), beta-blockers and mineralocorticoid/aldosterone receptor antagonists. Other than this, other treatments are used in certain patients that present particular symptoms include diuretics, angiotensin receptor neprilysin inhibitor, angiotensin II type I receptor blockers and I_f channel blockers. Where HFrEF can be clinically defined as left ventricular ejection

fraction (LVEF) <40%, the definition of HFpEF is less clear. In fact European Society of Cardiology (ESC) guidelines regard the diagnosis of HFpEF with LVEF ≥50%, and those with LVEF between 40-49% are considered to have mid-range ejection fraction. Therefore this perhaps shows that only a few patients with HfpEF receive the same types of treatments as HFrEF patients, whilst no treatment has been shown to be effective in reducing mortality or morbidity in HFpEF patients (Ponikowski et al., 2016).

1.2.8.1 Angiotensin-converting enzyme inhibitors (ACEi)

ACE plays a role in RAAS regulating sodium, extracellular fluid and cardiovascular function. ACE causes the conversion of angiotensin I to angiotensin II causing vasoconstriction. HF patients have activation of RAAS with upregulation of ACE resulting in increased amounts of angiotensin II. Angiotensin functions through two distinct receptors where one receptor causes vasodilation and natriuresis, the other functions to promote progression of HF causing vasoconstriction and sodium retention. ACEi has therefore been shown to improve cardiac output, decrease preload, causing vasodilation therefore decreasing blood pressure, promoting natriuresis and left ventricular remodelling (Demers et al., 2005).

Long-term treatment with enalapril (ACEi) has been shown to decrease the risk of death by 16% in patients with mild-to-moderate symptoms (McMurray et al., 2014). The CONSENSUS study (Cooperative North Scandinavian Enalapril Survival Study) showed that treatment with enalapril reduced mortality to 26% at 6 months in severe congestive HF patients. These changes were in conjunction with improved NYHA classifications and a reduction in heart size (Group, 1987).

ACEi is recommended for all symptomatic patients as well as those with asymptomatic left ventricular systolic dysfunction and up-titrated to the maximum dose to achieve the best level of RAAS inhibition (Ponikowski et al., 2016).

1.2.8.2 Beta-blockers

Beta-blockers (β-blockers) have become important in the treatment for chronic HF. β-blockers function by reducing the effects of the adrenergic nervous system (adrenaline and noradrenaline) and the RAAS which leads to chronic sympathetic effects on the heart. Blocking the sympathetic nervous system by the effects of β-blockers binding to

the β adrenergic receptors results in decreased blood pressure due to natriuresis and water retention (Barrese and Taglialatela, 2015).

The Metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure (MERIT-HF) and the Cardiac Insufficiency Bisoprolol Study II (CIBIS-II) both showed that β-blockers improved survival whilst decreasing the rates of hospitalisation in patients with NYHA class II, III, IV (Investigators, 1999, Merit, 1999). However, findings did suggest that although β-blockers were efficient in mild-moderate HF (NYHA class II-III), questions remained over the effects in more advanced HF (Beta-Blocker Evaluation of Survival Trial, 2001).

β-blockers are recommended to be given to clinically stable patients at low dose which is slowly up-titrated to the maximum tolerated dose. They are also recommended for patients with a history of myocardial infarction and asymptomatic left ventricular systolic dysfunction (Ponikowski et al., 2016).

1.2.8.3 Mineralocorticoid/aldosterone receptor antagonists

MRAs are involved with the effects of aldosterone on the heart. They function to antagonise the effects of aldosterone at mineralocorticoid receptors. Studies have shown that aldosterone has direct effects on the heart by decreasing coronary blood flow, increasing aortic blood flow and cardiac output. In cardiovascular disease, aldosterone effects have been shown in the development of myocardial fibrosis, a secondary event to inflammatory damage and vascular cytokine activation developed during hypertension. Binding of MRAs at the receptor inhibits sodium retention in the kidneys increasing diuresis. In HF they are often used alongside other drugs for increased effects of diuresis (Rocha and Williams, 2002).

The Randomized Aldactone Evaluation Study (RALES) showed that treatment with spironolactone in patients with HF showed a significantly reduced levels of morbidity and mortality (Rocha and Williams, 2002).

MRAs (spironolactone and eplerenone) are recommended in all symptomatic patients with LVEF ≤35%, however those with impaired renal function and serum potassium levels greater than 5.0 mmol/L require regular checks and monitoring (Ponikowski et al., 2016).

1.3 Myocardial infarction

Myocardial infarction (MI), commonly known as a heart attack, is a cardiac emergency that occurs when blood flow to the heart in one or more vessels is abruptly cut off leading to cardiac damage. It is characterised by symptoms including chest discomfort, nausea, shortness of breath and weakness (Anderson and Morrow, 2017).

1.3.1 Epidemiology and economic burden

MI is a worldwide emergency associated with substantial morbidity and mortality with an estimated 15.9 million sufferers worldwide in 2015 (Vos et al., 2016). The management of MI has dramatically improved over the past thirty years with mortality data showing a 50% decline in age-standardised mortality from MI in England (Bhatnagar et al., 2016).

1.3.2 Pathophysiology

Pathologically, MI is defined as cardiomyocyte death from an ischaemic insult. However, this is clinically a generic definition since many other conditions including heart failure and renal failure are able to cause non-ischaemic death of cardiomyocytes. It is however understood that the main underlying condition for MI occurrence is a rupture or erosion of an atherosclerotic plaque and occlusion from this is reflected by the severity of MI (Frangogiannis, 2015).

Atherosclerosis

The majority of patients presenting with MI have coronary atherosclerosis, such that the earliest atherosclerotic lesions can be commonly found in children which increase and develop into fibromuscular plaques, with age (Ross, 1986). Initial fatty streaks that trigger the process of atherosclerosis are thought to be driven by monocytes adhering to the surface of the endothelium. The plaque increases in size overtime through platelet adherence, aggregation and release of smooth muscle growth factors, and the adherence of macrophages in the lesion (Joris et al., 1983). This fibrous plaque results in abnormal transport of lipids which then become deposited in the plaque leading to

further release of macrophages, smooth muscle growth factors and fibrous cellular proliferation. Late-stage atherosclerosis is associated with necrosis and calcification within the plaque. The sequence of events for the initiating factors such as lipids, macrophages and platelets are currently unknown, however, it is possible that these could be selective in different individuals and at different times (Alpert, 1989).

Myocardial Ischaemia

Myocardial ischaemia is the result of an imbalance between oxygen supply and demand. Increasing atherosclerotic plaques and narrowing in the myocardial lumen does not cause a reduction in blood flow at rest. However, in circumstances where myocardial demand is increased for example due to exercise, this causes a restriction in blood flow which in turn inhibits the increasing demand for oxygen resulting in myocardial ischaemia (Frangogiannis, 2015). In ischaemia, aerobic respiration becomes inhibited and the levels of ATP rapidly decrease and normal systolic and diastolic function of the heart decline. This results in an increase in heart rate and systemic blood pressure due to the activation of the sympathetic nervous system. Prolongation or repetition of the ischaemic phase eventually leads to necrosis and waves of irreversible myocardial cellular injury (Alpert, 1989).

Ischaemic Necrosis

Necrosis is uncontrolled cell death and occurs by the opening of the mitochondrial permeability transition pore located within the inner mitochondrial membrane. The opening of the pore initiates the influx of water into the matrix resulting in mitochondrial swelling and necrosis. In ischaemic conditions, the opening of the pore is initiated by Ca^{2+} entry into the mitochondria causing loss of mitochondrial membrane potential and a reduction in ATP synthesis, and the influx of water resulting in swelling. The mechanism regulating the opening of the mitochondrial pores in necrosis are poorly understood (Frangogiannis, 2015, Baines et al., 2005).

1.3.3 Aetiology

There are many causes that lead to the slow progressive build of atherosclerosis. The common causes for MI are older age, male, smoking, high blood pressure, diabetes, and high total cholesterol levels. Many of these risk factors are included in risk models such as the Framingham risk score (O'Gara et al., 2012). Other causes include the lack of physical activity (7-12% cases), obesity (20% of coronary artery disease) and high chronic stress levels (Lee et al., 2012, Kivimäki et al., 2012).

1.3.4 Classification

Recent guidelines have introduced a classification by aetiology to show that MI occurs in a range of clinical cases. The introduction of sensitive cardiac biomarkers such as troponin is required with at least one value above the 99th percentile upper reference limit plus one of the following: symptoms of ischaemia, new ischaemic ECG changes, development of pathological Q-waves on the electrocardiogram, imaging evidence of loss of myocardium or wall motion abnormality using imaging techniques or identification of intracoronary thrombus by angiography or autopsy (Thygesen et al., 2018).

The Fourth Universal Definition of Myocardial Infarction identifies five types of MI: type 1, MI related to coronary thrombus; type 2, MI due to secondary ischaemia unrelated to acute athero-thrombosis; type 3, sudden cardiac death in patients with symptoms of MI and presumed new ischaemic ECG changes; type 4, MI associated with PCI (4a), stent thrombosis (4b) or MI restenosis (4c); and type 5, MI associated with coronary artery bypass grafting (CABG). Acute MI is classified with troponin concentrations being highly elevated and a dynamic change in ischaemia (Thygesen et al., 2018).

1.3.5 Acute myocardial infarction

Acute MI is the event that causes the most deaths or new cases of HF (Farah and Barbagelata, 2017). Acute MI is divided into ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction (N-STEMI), and further into the 5 types as described in 1.3.4. The initial mechanism for acute MI is rupture of

an atherosclerotic plaque and exposure of blood to the thrombogenic core. A total obstruction or occlusion of the plaque results in a STEMI, whereas a partial obstruction leads to a non-STEMI (N-STEMI) or unstable angina (Anderson and Morrow, 2017). The management of acute MI has improved over the years and continues to search for more effective treatments to control myocardial injury. Common areas of interest include the prevention of reperfusion injury by mechanical or pharmacological strategies (Spath et al., 2016).

1.3.6 Diagnosis

MI can be recognised by signs and symptoms before confirming with tests. Current tests include ECG to detect changes associated with contraction of the heart, imaging such as chest x-rays and echocardiography to visualise the size, shape and motion of the heart, and cardiac biomarkers such as troponin which is most commonly used and is a measure of injury (Califfi and Ohman, 1992).

For acute MI, diagnosis would be based on the detection of a rise and/or fall of troponin with at least one value above the 99th percentile of the upper reference limit coupled with at least one evidence of ischaemia from symptoms, changes in ECG such as ST segment changes, new left bundle branch block, or Q waves, evidence from imaging such as changes in the heart wall motion (O'Gara et al., 2012).

1.3.6.1 Signs and symptoms

The development to MI generally begins with myocardial ischaemia, lack of oxygen. Signs and symptoms are often non-specific and include chest pains and discomfort that often radiates towards the left arm, jaw, back and abdomen, dyspnoea and fatigue. Pain that usually lasts for more than 20 minutes is associated with acute MI. Chest pains can be accompanied by sweating, nausea, fainting or cardiac arrest (Thygesen et al., 2012). In addition to this, asymptomatic MI can occur and is associated with 22-64% of all infarctions. These are especially noticed in the elderly and are later discovered through diagnostic tests (Valensi et al., 2011).

1.3.6.2 Diagnostic tests

Patients suspected with MI are referred for clinical testing. Although there is no gold standard, myocardial injury is detected by elevations seen in biomarkers such as troponin and MB fraction of creatine kinase (CK-MB). In addition to this, in non-ischaemic cases, histological evidence of necrosis may be detectable. The ECG is an integral diagnostic test in patients with suspected MI and should be used swiftly. Non-invasive methods that employ imaging techniques such as MRI are often used (Thygesen et al., 2007). Troponin (cTnT or cTnI) is the preferred biomarker for MI diagnosis due to its high myocardial tissue specificity and clinical sensitivity (see section 1.5).

- Electrocardiogram (ECG), should be used immediately after presentation of signs and symptoms (within 10 minutes). Multiple ECG recordings are recommended due to dynamic changes in ECG waveforms during MI episodes. Such serial measurements in symptomatic patients are performed at 15-30 minute intervals. Changes in ECG waves allows the clinician to identify whether the MI is ST-elevated, allow to time the event, identify the infarct-related artery, estimate risk and prognosis and determine potential therapies (Thygesen et al., 2012, Zimetbaum and Josephson, 2003).
- CK-MB, creatine kinase was first introduced as a marker for MI in 1965 and has shown clinical sensitivity of 90% (Duma and Siegel, 1965). However, it has shown a lack of specificity. It is released within 12 hours after onset of acute MI, peaks at 24-36 hours and returns to normal concentrations at 48-72 hours. MB is the cardiac specific isoenzyme of CK and it was recommended to measure both total CK and CK-MB such that in some clinical settings, a CK-MB test is only required upon elevations in CK (Al-Hadi and Fox, 2009).
- Imaging tests such as magnetic resonance imaging (MRI) scans and echocardiography are useful non-invasive techniques for use in real-time. Echocardiography allows for imaging of myocardial thickness, thickening and motion at rest (Korosoglou et al., 2004). MRI allows for high spatial and moderate temporal resolution (Isbell and Kramer, 2005). Its full capabilities certainly using novel contrast agents is still in research.

1.3.7 Prognosis

Over the years prognosis of MI has improved, however HF remains one of the major acute complications post-AMI amongst other cardiovascular events including recurrent MI, stroke and death especially during the first 30 days. Age (>65 years) is associated with a greater risk of developing HF, and have a poorer prognosis (Torabi et al., 2014).

Evidence has also shown that individuals that survive from first and recurrent acute MI are at a greater risk of death compared with healthy individuals for at least 7 years. In addition to this, predisposing factors for a second episode of acute MI decreased over time after survival from a first MI (Smolina et al., 2012b). The MONICA study (monitoring trends and determinants in cardiovascular disease) showed evidence of improving trends in 12-year survival after acute MI is associated with the use of tailored treatment during admission and in the 12 months after the event (Briffa et al., 2009). Despite this, results have shown an overall improvement during the 2000s such that over half of the decline in deaths from acute MI was due to decreased event rate and just under half for improved survival at 30 days (Smolina et al., 2012a). Prevention and treatment have become revolutionary for these trends.

The American Heart Association recommend on seven factors (Life's Simple 7) to promote cardiovascular health and improve the impact of risk on incident. These include smoking, body mass index, physical activity, dietary quality, total cholesterol, blood pressure, and fasting glucose. Implementation of these in middle-age showed improvements in incidence and risk of adverse outcomes of MI in later life (Mok et al., 2017).

1.3.8 Treatment

MI requires immediate medical attention and the aims for treatment are to preserve the heart muscle and prevent further complications. Although MI is often divided into ST elevation MI (STEMI) or non-ST elevation MI (NSTEMI), the therapies are similar between the two. Commonly used drugs and interventions aim to unblock blood vessels, reduce enlargement of blood clot, reduce ischaemia, improve risk factors and prevent recurrences of MI (Reed et al., 2017).

1.3.8.1 Reperfusion and revascularisation

Reperfusion therapy is used to restore blood flow typically after MI and can include the use of drugs (thrombolytics and fibrinolytics) and/or surgery such as percutaneous coronary intervention (PCI). Reperfusion in acute STEMI has been shown to be the most important component phase of treatment where both drugs and surgery have shown to improve the outcome (Bassand et al., 2005). It is done to limit infarct size in addition with antithrombotic therapy, as studies showed rapid reperfusion was associated with lower mortality (Cannon et al., 2000). In contrast, in N-STEMI patients PCI is often employed to improve blood flow and prevent recurrent ischaemia. For N-STEMI patients, PCI is recommended to be achieved within 24 hours of N-STEMI, or up to 72 hours in low risk patients. PCI after 24 hours in N-STEMI patients has been associated with longer hospitalisation (Reed et al., 2017, Montalescot et al., 2009).

Fibrinolytic and thrombolytic agents are involved in the conversion of plasminogen to plasmin, resulting in the lysis of fibrin and dissolution of the clot. Fibrinolysis has been shown to reduce mortality by 29%, however trials suggest the preference of primary PCI to fibrinolysis (Reed et al., 2017).

1.3.8.2 Antithrombotic therapies

Antithrombotic therapy is thought to play a fundamental role in the management of MI due to the deposition of a thrombus. Both antiplatelet and anticoagulants appear to be necessary in the management of the whole spectrum of ACS, including MI. Although the exact effect of each drug is unclear, generally, the increasing potency of the drug is associated with a decreased risk of ischaemic events. However, excessive amounts of

antithrombotic agent leads to major bleeding complications, such that the balance between effective potency and risks remain a major challenge (Bhatt et al., 2014).

Activated platelets express the receptor for fibrinogen, essential for irreversible aggregation, and are heavily involved in the pathogenesis of MI. Antiplatelet agents work by inactivating the pathway (COX-1) that leads to further platelet recruitment and activation, resulting in reduced platelet function. For example, aspirin has a fast onset of action and is rapidly absorbed and reaches peak concentrations in the plasms after 30-40 minutes. It therefore serves as the most commonly prescribed drugs as an antiplatelet agent (Clappers et al., 2007). Antiplatelet agents such as aspirin are recommended to be given as soon as possible after diagnosis, and continued at a low daily dose. In addition to this, a combination of aspirin and $P2Y_{12}$ is also recommended for 12 months (Huber et al., 2014). Randomised trials have reported a reduction in death of MI of more than 50% in patients prescribed aspirin compared with placebo (Reed et al., 2017).

Anticoagulation agents such as heparin, enoxaparin or fondaparinux are recommended for all patients and can be discontinued after percutaneous coronary intervention. They are the choice of therapy for prevention and treatment of thrombosis. Thrombin is a potent activator of haemostasis and is able to potentiate clot formation. Anticoagulants can inhibit thrombogenesis by affecting the pathways associated with the clotting cascade or by targeting thrombin directly. Indirect inhibitors bind to plasma cofactors such as antithrombin. Commonly used, heparin, show rapid onset of antithrombotic activity and are considered indirect inhibitors as they bind to antithrombin, and upon activation of antithrombin, inactivation of coagulation factors and thrombin occurs (Alquwaizani et al., 2013). Many studies have shown the efficacy of anticoagulation agents alone and in combination with antiplatelet agents. The use of unfractionated heparin has been ever present for over 20 years with a number of studies showing the addition of unfractionated heparin to aspirin during acute phase reduces MI and mortality (Reed et al., 2017). The use of warfarin with aspirin was associated with a significant decrease in the rate of ischaemic complications but an increase in major bleeding compared with aspirin alone (Bloemen et al., 2017).

1.4 Processed B-type natriuretic peptide molecular forms

Following the discovery of atrial natriuretic peptide (ANP) by de Bold in 1981, BNP was identified in 1988. Initially named Brain Natriuretic Peptide, it was first noticed in porcine brain as a novel peptide of 26 amino acid residues showing very similar amino acid sequence, structure and function to that of ANP. Although BNP was first isolated in the brain; Maekawa et al showed using a porcine model that the highest concentration was actually found in the cardiac atrium, and subsequent studies showed that it is predominantly expressed in the ventricle. A third member of the natriuretic peptide family, CNP, along with BNP was shown to be co-fractionated with ANP. CNP differs from BNP and ANP in that it is primarily expressed in the central nervous system and the vasculature, rather than the heart (Maekawa et al., 1988, Sudoh et al., 1988, Suzuki et al., 2001).

1.4.1 Biochemistry of natriuretic peptides

The major biochemical and physiological comparisons for the three common natriuretic peptides are shown in Table 1.3.

Table 1.3 Summary of the major biochemical and physiological actions of ANP, BNP and CNP

	ANP	BNP	CNP
Also known as	ANF	Low-molecular weight BNP BNP-32 BNP 1-32	
Gene	*NPPA*	*NPPB*	*NPPC*
Amino acid residues	28	32	22
Receptors	NPR-A, NPR-C	NPR-A, NPR-C	NPR-B, NPR-C
Precursor	PreproANP (151 amino acids)	PreproBNP (134 amino acids)	PreproCNP (126 amino acids)
Prohormone	proANP (126 amino acids)	proBNP (108 amino acids)	proCNP (103 amino acids)
Storage/Location	Atrial granules	Mainly secreted from the ventricles by rapid gene expression and *de novo* synthesis.	Located in numerous tissues including CNS, renal tubular cells and vascular endothelial cells
Synthesis	Cardiomyoctes (mainly atrial)	Ventricular cardiomyocytes	Vasculature, CNS
Circulating fragments	NT-proANP, ANP (Active)	NT-proBNP, BNP (Active)	CNP (22 amino acids Active), CNP (53 amino acids)
Plasma half life	~3 minutes	~20 minutes	~2.6 minutes
Factors stimulating release	Increase in filling pressure of the heart and stretch of atrial wall. Neurohormones (endothelin-1, anti-diuretic hormone, catecholamines)	Increase in filling pressure of the heart and stretch of ventricular wall. Neurohormones (endothelin-1, anti-diuretic hormone, catecholamines)	
Major Actions	Natriuresis, Diuresis, Vasodilation	Natriuresis, Diuresis, Vasodilation	Weak vasorelaxant and natriuretic peptide, suppress cell proliferation, exerting antifibrotic and antihypertrophic effects

ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide

(Potter, 2011, Boomsma and van den Meiracker, 2001, Kalra et al., 2001, Valle et al., 2013, Dietz, 2005, Brunner-La Rocca et al., 2001)

1.4.2 Structure and function

The three natriuretic peptides (NP) contain an intramolecular and highly conserved 17 amino acid ring region with a disulphide bond, involved in receptor binding (Figure 1.2). This homology is greater than in other regions, such as the N-terminal and C-terminal regions. CNP does not contain a C-terminal and is therefore different in structure and function. It is evident that CNP is lacking a C-terminal tail, whereas ANP and BNP contain five and six amino acids in their C-terminal regions respectively. The N-terminal regions of all three structures differ in the number of amino acid residues. The structures of both ANP and BNP are very similar (Shimekake et al., 1992).

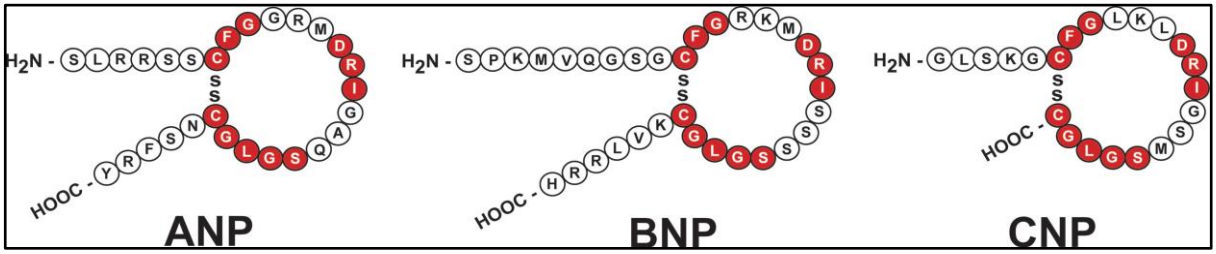


Figure 1.3 Primary structure of the three natriuretic peptides; ANP (28 amino acids), BNP (32 amino acids) and CNP (22 amino acids). Homologous amino acids are part of the highly conserved ring region and are highlighted in red. The disulphide bond is also showing forming part of the ring region. Image taken from (Goetze, 2012)

NPs are cardiac hormones, and the roles of ANP and BNP are central to cardiovascular homeostasis. As the name suggests, they induce a natriuretic activity as well as diuretic and hypotensive activity. Their functions include; regulation of salt and water intake, controlling blood pressure, relaxing the vascular smooth muscle, inhibiting the RAAS and inhibiting the secretion of anti-diuretic hormone (ADH) in attempts to reduce body fluid and decrease blood pressure (Ala-Kopsala et al., 2004).

As a neurohormone, it is also thought that the effects of NPs extend to the CNS and its involvement in neuroendocrine control; however there is a lack of research to support this (Imura et al., 1992). CNP does not exert natriuretic effects, and is suggested to inhibit vascular smooth muscle cell proliferation in rat models by increasing cGMP

levels, and by the same mechanism it inhibits cardiac myocyte hypertrophy. CNP is a weaker hormone in exerting vasorelaxant and natriuretic effects; however it is stronger in antifibrotic and antihypertrophic effects suggesting its role as an autocrine regulator inhibiting cardiac fibrosis (Furuya et al., 1994, Horio et al., 2003, Soeki et al., 2005, Tokudome et al., 2004).

1.4.3 Synthesis and processing

BNP is derived from a precursor molecule encoded by the NPPB gene, whereas the precursor molecules for ANP and CNP are encoded by NPPA and NPPC, respectively. The expression of NPPC is most abundant in the nervous system and endothelial cells, NPPA and NPPB are cardiac hormones with their production central to the atria and ventricles, respectively. Therefore NPPA and NPPB are closely linked on human chromosome 1p36, consisting of three exons and two introns, with the latter exon containing most of the coding sequence, suggesting that ANP and BNP may have evolved from a common ancestor. Transcription of the NPPB gene occurs in response to cardiac overload and increase in pressure and, unlike ANP, proBNP expression is upregulated prior to secretion and the turnover of BNP mRNA is significantly faster. At a post-transcriptional level, the mRNA consists of a conserved repeating sequence of AUUUA in the 3'-UTR. It is thought that the AU-rich sequences such as those in NPPB are central to accelerating degradation of BNP mRNA as seen in genes for cytokines and growth factors with the AUUUA motif. Through the rapid induction of gene expression of BNP from the ventricles, it is suggested that BNP serves as an emergency measure against cardiac overload, whilst the clearance of BNP has been shown to be slower than the clearance of ANP (Arden et al., 1995, Nakagawa et al., 1995, Szabó, 2012, Wilson and Treisman, 1988).

Primarily synthesised in the membrane granules of the ventricles and produced by cardiomyocytes, BNP begins as a 134 amino acid pre-pro BNP polypeptide including a signal peptide. The signal peptide is then removed and the resulting 108 amino acid prohormone is cleaved by proteolytic enzymes, to give rise to two processed peptides; the mature physiologically active 32 amino acid BNP formed from the carboxyl terminus of pro-BNP and the physiologically inert 76 amino acid NT-pro-BNP (Figure 1.3)

(Fujimoto et al., 2013, Shimizu et al., 2002). It is also understood that cardiac fibroblasts produce BNP. Similarly, ANP has also been reported to be synthesised in cardiac fibrocytes in sheep after MI. Furthermore, BNP was shown to be up-regulated by TNF-α, which is involved in ventricular remodelling. These findings suggest that BNP could be involved in regulating ventricular remodelling by controlling the function of cardiac fibroblasts, especially in disease states (Tsuruda et al., 2002).

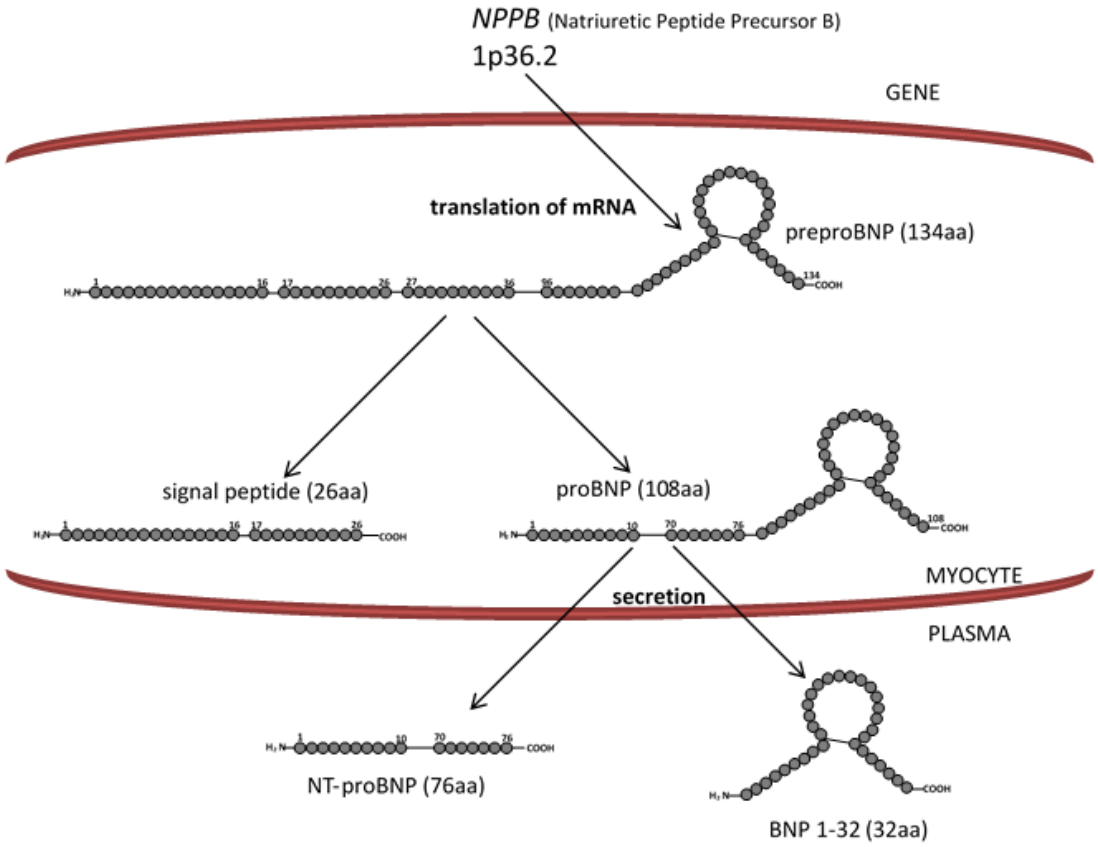


Figure 1.4 Showing BNP processing from pre-proBNP which is cleaved to form proBNP and a signal peptide. ProBNP is then cleaved to form NT-proBNP and BNP 1-32.

In contrast ANP is synthesised from a precursor molecule, pre-proANP from the translated mRNA. The removal of 25 amino acid residues from this 151 amino acid molecule results in proANP, which is then cleaved into the carboxyl terminal active form, 28 amino acid ANP, and the N- terminal 98 amino acid inactive NT-pro-ANP (Nakao et al., 1992). Mukoyama et al demonstrated that BNP differs from ANP in secretion, synthesis and clearance, whilst showing that ANP and BNP are part of a dual

natriuretic peptide system for cardiac control with ANP secretion from the atrium and BNP from the ventricles. BNP was shown to remain in circulation longer than ANP, explaining why HF patients have a slow clearance of BNP, with further secretion from the heart and increased BNP in the plasma (Mukoyama et al., 1991).

It was found that BNP is secreted from the heart at a faster rate than ANP in patients with MI, where results showed that within hours of onset BNP levels were higher than 100 times the normal level of BNP compared to the increase of ANP levels (Mukoyama et al., 1991). Although BNP is secreted more rapidly than ANP, ANP is stored in granules and can be secreted by stimulation of these granules which tend to act quickly in acute states. This shows secretion is based on two factors; the rate of synthesis and the rate of secretion (Naruse et al., 1994).

1.4.4 Processing enzymes

Corin and furin, are protein convertases and thought to be proBNP processing enzymes. Corin is expressed in cardiac myocytes and has been shown to act as a proANP convertase and therefore is involved in blood pressure regulation. It is thought that it contains a phosphorylation site in the cytoplasmic domain, which could be the site for phosphorylation whilst the cell surface would be the site for signalling (Hooper et al., 2000). It is expressed in the same cardiomyocytes as ANP, and cleaves the precursors upon secretion. Corin has been shown to adapt to the condition present in the heart, where it can become several molecular forms reflecting the zymogen state and active state. Semenov et al showed that it can be inhibited and prevent cleavage of proBNP with the presence of *O*-glycosylation close to the cleavage site. The preferred cleavage site for corin is at Arginine/Lysine residues towards the N-terminus, Proline/Phenylalanine/Glycine at the second position and a neutral amino acid at the third position. This has also been shown for furin (Gladysheva et al., 2008, Semenov et al., 2010). Corin deficiency in mice is reported to contribute to hypertension, by preventing the processing of natriuretic peptides, whilst similar results have occurred in human subjects. The expression patterns show that in humans, there were similar levels in failing and non-failing hearts in the atria whereas in the ventricle the failing hearts was more greatly expressed. This suggests that corin expression in patients with

failing hearts is a slow step preventing sufficient processing of natriuretic peptides when required, but as natriuretic peptide levels increase, corin levels also increase for processing (Chen et al., 2010).

Furin is a protein convertase reported to cleave many proteins since it is widely expressed throughout the tissues and located primarily in the trans-Golgi network. It was shown to process BNP from proBNP, with the preferred cleavage sequence site of -Arginine-X-X-Arginine ↓X-, as found in proBNP, therefore identifying this as a possible substrate and cleavage site for BNP processing (Semenov et al., 2010). Furin is an intracellular endoprotease containing an abundance of Golgi apparatus, and has been reported that its expression increases along with natriuretic peptides in the atria of patients of early stage heart failure, in contrast to corin where the expression decreased (Ichiki et al., 2013). The processing of proCNP was investigated with results showing that recombinant corin did not enhance proCNP processing; however processing was inhibited when a furin inhibitor was used. This suggests that in proCNP processing, furin cleaves to form CNP, whereas corin is not used (Wu et al., 2003). Furin was also found to be co-expressed with BNP in the atria and ventricle shortly after myocardial infarction, suggesting its role to rapidly process BNP, induce natriuresis and lower blood pressure (Sawada et al., 1997).

Whether corin is a processing enzyme to truncate the precursor or NP is still unclear. Results from different studies have confirmed the processing of proBNP by corin results in the formation of BNP 4-32 (Semenov et al., 2011). The expression patterns of both furin and corin show that although they carry out similar functions they show opposing protein expression during HF. The significance of the effects in HF and processing of BNP remains to be established.

1.4.5 Receptors

NPs function via natriuretic peptide receptors (NPR). Currently, three receptors have been identified in numerous tissues. NPR-A and NPR-B are signal transducers consisting of transmembrane glycoproteins displaying their effects through ligand-dependent intrinsic guanylyl cyclase activity, whose activity is affected by glycosylation and phosphorylation of NPR. Upon binding, these receptors generate the second messenger, cGMP from GTP (Figure 1.4). NPR-A has been identified as the preferred binding site for ANP and BNP, with ANP having higher affinity. NPR-B therefore is a preferred binding site for CNP followed by ANP and then BNP. The NPR-C, structurally distinct from NPR-A and NPR-B, is not associated with cGMP production, lacking the cyclase domain, and could therefore possibly function as a clearance peptide receptor binding with equal affinity for all peptides (Noubani et al., 2000, Chang et al., 1989). The NPR-C, consisting of a 37 amino acid intracellular domain sequence has been shown to exert its effects by guanine nucleotide-binding proteins (G proteins), whilst it has also been shown to inhibit adenylyl cyclase in platelets expressing NPR-C (Murthy et al., 2000).

However, in an experiment by Suga et al, it was discovered that ANP bound to NPR-C with a greater affinity, followed by CNP and lastly BNP in humans and rats. This information provides evidence of the rate of degradation between ANP and BNP, which has been previously shown to be slower in BNP due to BNP containing the AUUUA motif (Suga et al., 1992, Wilson and Treisman, 1988).

NPR-A is most abundantly expressed in the vasculature, kidneys and adrenals. NPR-B is most abundantly expressed in the brain, outlining its role as a receptor for CNP and its role in neuroendocrine regulation, however, NPR-B can also be found in the adrenals and kidneys. The distribution of NPR-C is within most tissues expressing the guanylyl cyclase receptor, including the kidneys. Its expression is more abundant than NPR-A and NPR-B (Matsukawa et al., 1999). It was shown by Ritter et al that in the kidneys, NPR-B is expressed in the inner medullary collecting duct, highlighting the failure of CNP to exert natriuretic effects, whereas the absence of NPR-A in the cortical collecting tubule

and presence of NPR-B shows the possible role of CNP in acid-base homeostasis (Ritter et al., 1995).

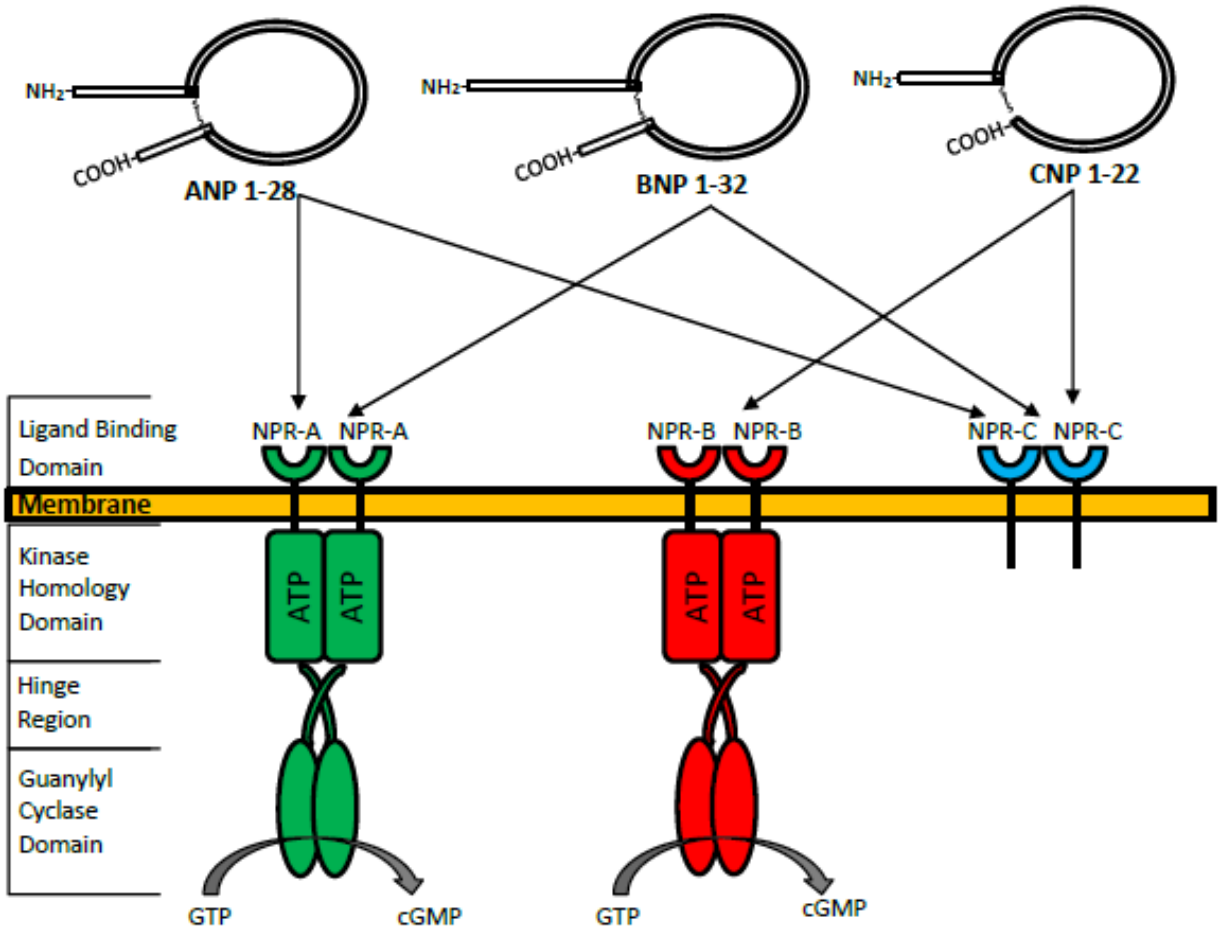


Figure 1.5 Illustration of natriuretic peptide receptors. NPR-A, natriuretic peptide receptor-A, NPR-B, natriuretic peptide receptor B and NPR-C, natriuretic peptide receptor C. Binding of NP to their respective receptors is shown by solid arrows.

1.4.6 Metabolism and clearance

Metabolism of ANP and BNP is characterised by two major pathways: enzymatic degradation by NEP and NPR-C exerting its effects as a clearance receptor by endocytosis and lysosomal degradation. NEP 24.11 is present in many tissues throughout the body, but mainly distributed at the brush border in the proximal tubule of the kidneys. NPR-C is within most tissues expressing the guanylyl cyclase receptor. Both of these showed an equal contribution in the metabolism of ANP and BNP in sheep with heart failure (Rademaker et al., 1997).

It was discovered by Maack et al that neprilysin, a zinc metalloprotease, cleaves NPs by opening the ring structure, where NEP binds to the ring structure of ANP with approximately 99% affinity and inactivating it (Maack et al., 1987). It was shown that in pig BNP, the point of attack by NEP was at the Serine-Leucine bond at amino acid residues 20-21, however in human BNP the point of cleavage was at Methionine-Valine 4-5 amino acid residues, releasing the N-terminal peptide before a second attack at positions 17-18 (Arginine-Isoleucine) (Kenny et al., 1993).

NPR-C functions by the binding of natriuretic peptides, which are then hydrolysed by lysosomes and the receptor is then recycled to the cell surface. These are the most abundant of the receptors and are located in numerous tissues in the body. It has been reported that studies have shown an increase in mRNA expression of NPR-C in damaged hearts. The results from this have suggested that NPR-C up-regulation in HF explains increased ANP clearance. The results from NP clearance suggest the possible role of treatment for heart failure through inhibition of NEP (Andreassi et al., 2001).

A summary table showing the major biochemical and physiological comparisons for BNP, NT-proBNP and proBNP is shown in Table 1.4.

Table 1.4 Summary of the major biochemical and physiological actions of BNP, NT-proBNP and proBNP (Hobbs and Mills, 2008, Cowie et al., 2003)

	proBNP	NT-proBNP	BNP
Also known as	High-molecular weight BNP		Low-molecular weight BNP BNP-32 BNP 1-32
Amino acid residues	108	76	32
Hormonal activity	Low activity (6- to 8-fold lower activity compared to BNP)	Inactive	Active
Secretion	Released from ventricular myocytes following preproBNP cleavage	Cleaved from proBNP	Cleaved from proBNP
Plasma Half Life	Unknown	~ 120 minutes	~20 minutes
Plasma Stability	Unknown	Up to 2 days at room temperature	4 hours at room temperature
Clearance	Unknown	Renal	Neutral endopeptidase (NEP) Receptor mediated clearance (NPR-C)
Cut off value to rule out heart failure		400pg/mL	100pg/mL

1.4.6 Role in disease

BNP was described as a cardiac biomarker more than two decades ago. Owing to the role of BNP as a natriuretic, diuretic and vasodilating hormone, the levels of BNP change in disease states especially in the cardiovascular and renal system.

1.4.6.1 Renal system

In a healthy individual, NPs function to stimulate natriuresis and diuresis, primarily by acting at the glomerulus and collecting duct. It has been shown to cause dilation of the afferent arterioles and constriction of the efferent arterioles resulting in increased glomerular filtration rate (GFR), whilst decreasing blood pressure. The increase in GFR and urine flow showed levels of five-fold increase in urine flow and increase excretion rates of sodium at two-fold amongst other electrolytes when human ANP was infused in healthy volunteers, outlining the effects of natriuretic peptides on the kidneys (Weidmann et al., 1986).

In disease states, renal impairment and cardiac disease are associated with RAAS as well as BNP, where it has been shown that following renal impairment the effects on the heart occur only a few hours after. Patients with acute kidney injury have been shown to have increased levels of BNP, increasing for 48 hours post-injury (de Cal et al., 2011). Renal dysfunction has been shown to increase the levels of both BNP and NT-proBNP, with progressive renal disease resulting in increasing concentrations and decreased clearance from the kidneys. It was also found that renal function has less impact on BNP clearance compared to NT-proBNP clearance (Srisawasdi et al., 2010).

1.4.6.2 Cardiovascular system

Cardiovascular effects of NPs are primarily to act as a vasorelaxant to lower blood pressure. In the healthy heart, BNP secretion mainly occurs in the left ventricle, whilst ANP secretion occurs from the atria in response to myocardial stretch. However, during left ventricular dysfunction, both natriuretic peptides are secreted from the left ventricle and this secretion increases in line with severity (Yasue et al., 1994). Therefore, with reference to ANP secretion, it is regulated by the right and left arterial and ventricular pressures, dependent on the physiological state of the heart.

In HF, NP levels increase in order to fulfil the demand of the heart and reduce blood pressure, where secretion levels of BNP are often greater than ANP. These levels have also been noticed in renal impairment, MI and hypertension. Results have shown that over-expression of BNP in transgenic mice results in lower blood pressure, vascular resistance and decreased effect of glomerular injury (Kasahara et al., 2000). In HF, BNP levels are reported to be inversely correlated to left ventricular systolic function, and increases are related to diastolic function (Salustri et al., 2009). Alternatively in HF, it was reported that some patients may have NP deficiency where the proBNP, which is detected as a substantial portion immunoreactive BNP (irBNP), shows less activity than BNP. This suggests the reason why some patients, although possessing high levels of irBNP in the plasma, still suffer from severe symptoms due to the majority of the irBNP being proBNP (Liang et al., 2007).

1.4.7 Molecular forms

BNP is part of the carboxyl terminus of pro-BNP. Studies have identified two major forms of BNP in the plasma; high molecular weight and low molecular weight thought to correspond to pro-BNP and BNP, respectively. Although the metabolism of the amino terminus and precise structure of circulating BNP is unknown, it is understood that the disulphide-bond mediated ring structure and carboxyl terminus are stable. Shimizu et al reported that BNP incubation in whole blood in a siliconised glass tube showed the release of two amino acid residues from the amino terminus (Serine and Proline) and analysis by reverse-phase high-performance liquid-chromatography confirmed a small amount of BNP 3-32 was found in the plasma of HF patients (Shimizu et al., 2002).

Niederkofler et al identified numerous molecular forms of BNP in the plasma of HF patients using protease inhibitors. The most common form found was BNP 3-32 and was thought to be due to the actions of DPPIV (Figure 1.5). Other forms included; BNP 4-32, BNP 5-32, BNP 5-31, BNP 1-25 and BNP 1-26. The actions of these shorter molecular forms in their ability to generate cGMP showed no difference compared to BNP, however, it is expected that the in vivo actions within the body would produce lower levels of natriuretic activity (Niederkofler et al., 2008). Niederkofler et al has shown that there are three major molecular forms of BNP; BNP 3-32, 4-32 and 5-32, and the further forms found by Niederkofler et al were found at very low concentrations and not consistently identified in studies by other authors.

Common molecular forms were also found in ischaemic heart disease. BNP forms 3-32, 4-32 and 5-32 were identified as well as low levels of BNP. It was also noticed that lower levels of BNP 5-32 were seen in conditions of restenosis at the time of follow up compared to BNP 3-32, suggesting the role of BNP 5-32 in the pathophysiology of restenosis (Fujimoto et al., 2013).

In porcine heart, the concentration of irBNP was much less than ANP in the atria and plasma. However it was found that three molecular forms of irBNP were present weighing approximately 3K Daltons, showing the possibility that BNP-26, BNP-29 and BNP-32 could circulate in the blood (Aburaya et al., 1989).

BNP 3-32 was shown to be the results of BNP cleavage by purified DPPIV when BNP was incubated at room temperature in EDTA-plasma. DPPIV is a cell surface protease found in plasma and cleaves many peptides, particularly from the amino terminal in peptides that contain a Proline or Alanine as the second amino acid; a proline is found in this region for BNP 1-32 and proANP 1-30. Therefore, proBNP can also be cleaved by DPPIV to proBNP 3-108 (Brandt et al., 2006). In patients with HF, results have shown that low levels of BNP are in circulation in addition to BNP 3-32, BNP 4-32 and BNP 5-32, as analysed by mass spectrometry. These results suggest that it is the sum of the molecular forms that contribute to clinical BNP measurements, and not the intact BNP alone. It was noticed that clinical BNP correlated best with mass spectrometry results of the molecular forms, whilst intact BNP delivered a poor correlation (Miller et al., 2011).

Catabolism of BNP and its products was investigated by Pankow et al and a multimeric metalloprotease was identified as meprin A. Expressed in the brush border of the renal tubules, in mouse models it was shown to truncate from the N-terminal of BNP to BNP 7-32, conserving its biologic activity. Here it was shown that meprin A initiates BNP degradation to BNP 7-32, thought to be identical to the 26 amino acid residue BNP first identified by Sudoh et al, whilst also being catabolised by NEP. This showed that inhibiting meprin A in murine models could increase the levels of circulating BNP in the plasma, identifying a potential therapeutic target to increase BNP (Pankow et al., 2007).

Alternatively, BNP 8-32 has also been identified in canine models with meprin A cleaving BNP, with the resulting BNP 8-32 having similar haemodynamic effects such as vasodilation but reduced renal effects including natriuresis and diuresis. It was found that cardiorenal actions increased more with irBNP compared to BNP 8-32, although this is thought to be due to the antibody directed towards the epitope on the N-terminus of BNP, which is absent in BNP 8-32. This could also be due to BNP 8-32 being degraded more rapidly and having reduced bioactivity, where NEP, would only degrade BNP after cleavage by meprin A (Boerrigter et al., 2009).

However, both of these studies identifying BNP 7-32 by Pankow et al and 8-32 by Boerrigter et al have limitations. Firstly they were both conducted in animal models, therefore it is difficult to fully reflect these findings in humans. Secondly, these molecular forms have yet to be noticed in the human body and it is unknown whether

they exist in circulation. Finally these experiments were conducted in vitro, and the effects in vivo are difficult to determine. These two identified forms are rare in terms of their identification in studies, however, with further research into the role of meprin A; the inhibition of meprin A could form the basis of a therapy to up-regulate the activity of BNP.

Molecular forms, even in commercial assays are potentially detected as opposed to the active BNP. It was shown that two commercial testing kits; Biosite Triage and Shionogi detected BNP 3-32 in addition to BNP. Results also showed that cGMP activity in BNP 3-32 and 1-32 were similar in fibroblasts and cardiomyocytes, whereas proBNP and NT-proBNP showed no activity. It has also been reported using mass spectrometry analysis, BNP 1-32 was absent from the spectrum, further suggesting the possibility that molecular forms tend to be detected in commercial assays. This study did however detect a molecule of 8-kDa, that was eluted with BNP, but did not correspond to it. These results provide evidence of the possibility that other molecular forms of BNP are detected in commercial assays and therefore may be present in circulation (Heublein et al., 2007).

1.4.8 Conclusion

BNP, since its discovery, has been used as a diagnostic marker to rule out HF. Since it is involved in natriuresis, diuresis and cardiovascular haemostasis, its effects are exerted in the renal system also. Its structure and function have been established in studies, however the processing, metabolism and clearance are not fully understood. Despite it being a diagnostic marker in HF, commercial assays have identified that BNP levels detected are in fact molecular forms or truncations of BNP, rather than BNP itself. Common forms identified include BNP 3-32, 4-32 and 5-32, and their function, metabolism and processing are currently unknown. However, their detection in circulation may allow in the future for their use in diagnosis, prognosis and therapy.

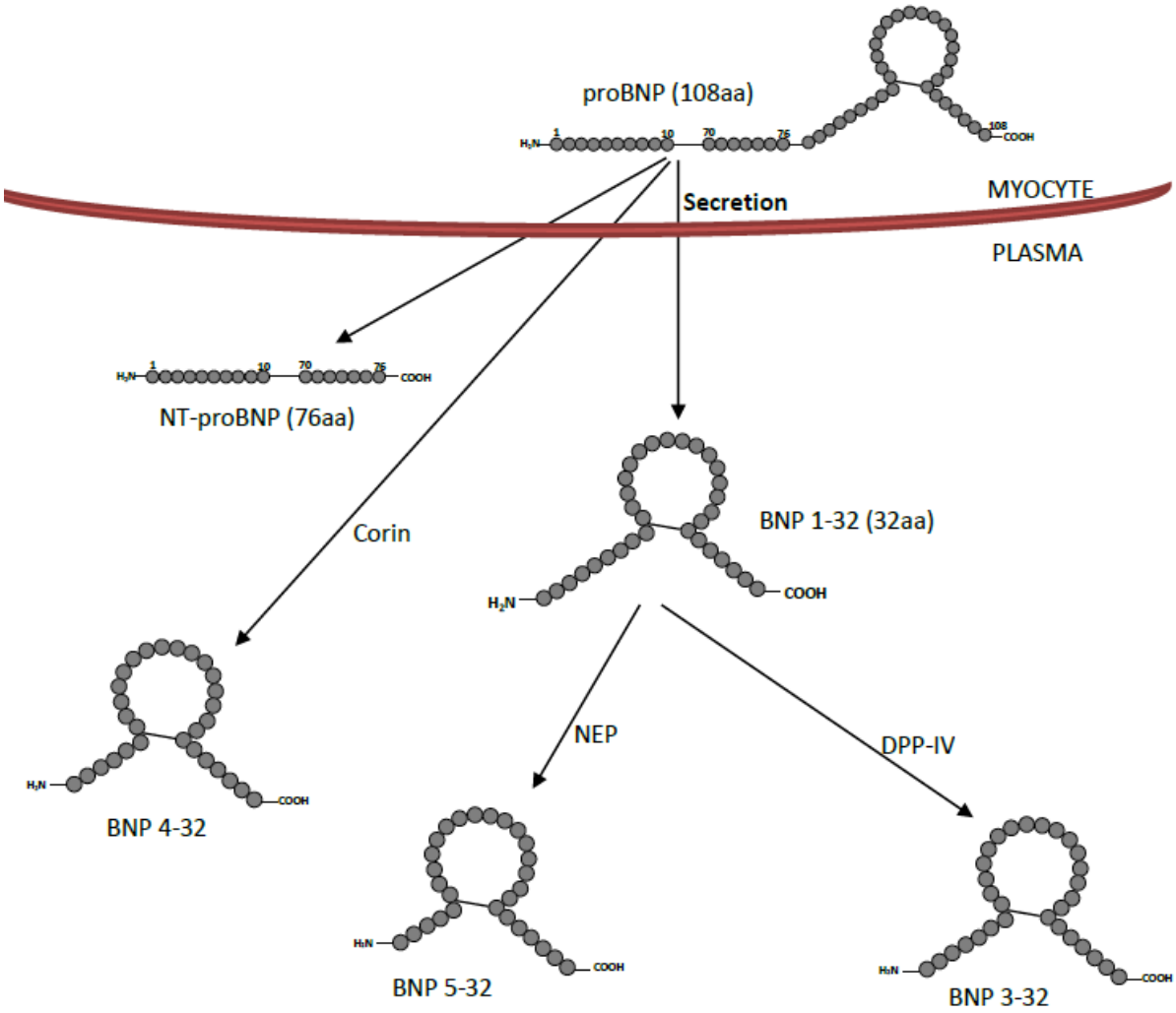


Figure 1.6 Depicting the proposed route of processing, location and synthesis showing the possible proteases involved in the formation of BNP molecular forms BNP 5-32 (NEP, (Pankow et al., 2007)), BNP 4-32 (Corin, (Semenov et al., 2010)) and BNP 3-32 (DPP-IV, (Brandt et al., 2006)) from their parent molecule

DPP-IV, dipeptidyl peptidase-IV; NEP, neutral endopeptidase

1.5 Proteomics

Proteomics was a term first coined in the 1990's, although protein studies that can be described as proteomics have been known to originate in the 1970's. Proteomics can be defined as the large-scale study of proteins as part of the proteome. The proteome involves the entire set of proteins expressed by a genome, cell, tissue or organism at a certain time since for every cell will change over time in response to certain stimuli. The study of proteomics covers protein expression, localisation, interaction, domain structure, modification and activity (James, 1997, Graves and Haystead, 2002).

Numerous method are employed in proteomics but are generally based on immunoassays using antibodies or mass spectrometry. Methods relying on antibodies include ELISA, Western blotting, immunoblotting and 2D-PAGE. Antibody-free methods are based on mass spectrometry and involve using either a 'bottom up' or 'top down' approach or analysis using protein chips. Hybrid methods are commonly used, involve antibodies to capture the protein of interest and analysis using mass spectrometry, hence the name for this technique Mass Spectrometry Immunoassay (MS-IA). Chromatography tends to be used in proteomics to purify the sample prior to analysis giving better sensitivity, and due to the advances in science chromatography is often coupled to mass spectrometers (LC-MS) combining the advantages of the two technologies for higher molecular specificity and detection sensitivity (Hirsch et al., 2004).

1.5.1 Mass spectrometry

Mass spectrometry is a widely used analytical technique used in protein research replacing older techniques primarily due to faster analysis, higher sensitivity and the ability to provide accurate mass and charge of the proteins present. Its applications are widely used and cover fields such as forensics, food industry, sports' doping and pharmaceutical and medical research. Mass spectrometry also allows multiple forms of complex analysis from one sample and by combining with LC (forming LC-MS) provides increased sensitivity due to the physical separation of liquid chromatography and analysis by mass spectrometry as shown in clinical toxicology and drug screening. Mass spectrometry over time has progressed and allowed other configurations to be added

for complex studies including the addition of a quadrupole-time of flight (Qq-TOF-MS) and tandem MS (MS/MS) for the analysis of gemini surfactants in drug delivery (Chindarkar et al., 2014, Donkuru et al., 2014).

Regardless of the mass spectrometer, all mass spectrometers involve three key aspects beginning with ionisation, mass analyser and detection. The ion source converts analyte molecules into gas-phase ions, a mass analyser separates and measures these ions according to their mass-to-charge (*m/z*) ratio, and a detector that detects and records the number of ions at the corresponding *m/z* ratio (Figure 1.6) (Aebersold and Mann, 2003).

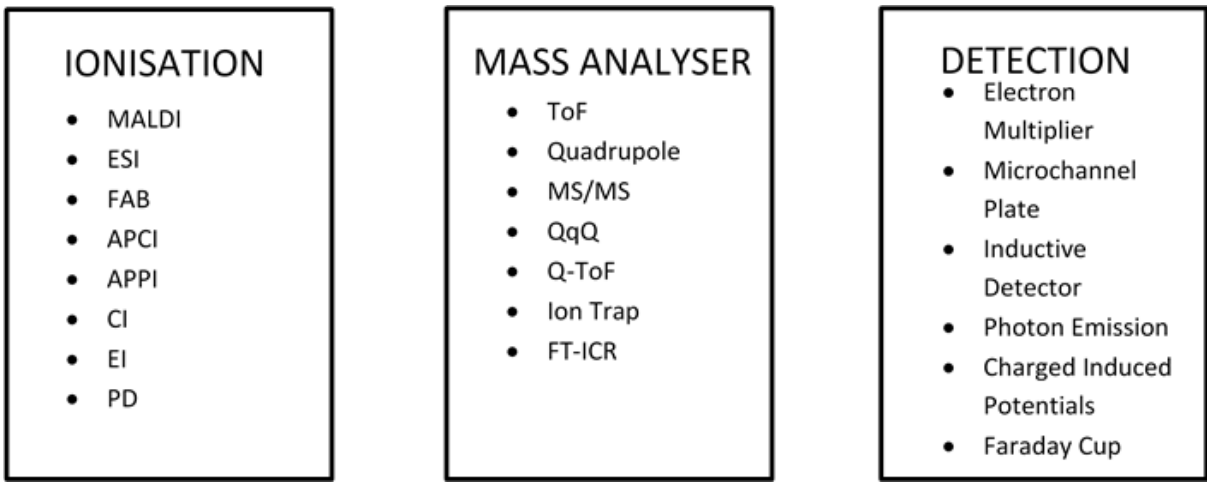


Figure 1.7 The three aspects common in all mass spectrometers consist of ionisation, mass analyser and detection. Listed are the commonly used components in each of the three domains.

APCI, Atmospheric pressure chemical ionisation; APPI, atmospheric pressure photoionisation; CI, chemical ionisation; EI, electron ionisation; ESI electrospray ionisation; FAB , fast atom bombardment; FT-ICR, Fourier transform ion cyclotron resonance; MALDI, matrix-assisted laser-desorption ionisation; MS/MS, tandem mass spectrometry; PD, plasma desorption; QqQ, triple quadrupole; Q-ToF, quadrupole-time-of-flight; ToF, time-of-flight

1.5.2 Ionisation

Ionisation is a critical part of mass spectrometry where the analyte molecules are converted into gas-phase ions suitable for the mass analyser.

There are numerous ways of ionisation and they depend on their subsequent application carrying their own advantages and disadvantages. Ionisation can be divided into two categories; hard ionisation and soft ionisation. Hard ionisation refers to the process where large amounts of energy is transferred to the analyte resulting in large amounts of fragmentation and ions with a smaller *m/z*. Soft ionisation is the more common process used in proteomics and refers to minimal internal energy transferred to the analytes resulting in little fragmentation and a higher *m/z*. Ionisation techniques include fast atom bombardment (FAB), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI), chemical ionisation (CI), electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI). Table 1.5 explores the differences between the different types of ionisation. Two of the most common ionisation techniques used in mass spectrometry proteomics are soft ionisation methods; ESI and MALDI (El-Aneed et al., 2009).

Table 1.5 Summary of currently used ionisation techniques in mass spectrometry in order of lowest mass range to high

	Method	Mass range	Analytes	Sample introduction
EI	Hard ionisation forming fragment ions	Less than 1000 Daltons	Volatile and thermally stable	Gas chromatography
CI	Soft ionisation forming molecular ions	Less than 1000 Daltons	Volatile and thermally stable	Gas chromatography
APCI	Soft ionisation, forming ions with few fragmentations	Up to 1500 Daltons	Compounds of low-medium polarity	Sample solution introduced at 200-2000 µL/min flow rate in a nebuliser that sprays the sample under atmospheric pressure
APPI	Soft ionisation	Up to 1500 Daltons	Non-polar compounds	Sample solution vaporised by heated nebuliser resulting in gaseous sample molecules interacting with photons emitted from a discharge lamp
FAB	Soft ionisation,	Less than 5000 Daltons (200-2000 Daltons)	Organometallic compounds	Liquid chromatography, sample mixed in viscous matrix and injected directly
PD	Soft ionisation	Up to 100,000 Daltons	Biomolecules	Sample coated with high energetic fragment, notably Californium-252. Fission fragments from Californium-252 decay volatilise and ionise sample.
MALDI	Soft ionisation, singly charged ions	Beyond 500,000 Daltons	Biomolecules organic and inorganic compounds	Sample co-crystallised within UV-absorbing matrix
ESI	Soft ionisation, multiply charged ions	Very low to very high mass (up to 100 kDaltons)	Biomolecules, organic and inorganic compounds	Sample solution introduced at 1-1000 µL/min into a spray capillary. High voltage applied (3-6kV).
NanoESI	Soft ionisation, multiply charged ions	Very low to very high mass (up to 100 kDaltons)	Typically used for peptide and protein analysis	Sample solution introduced at nanoliter/minute range (~25 nL/min) into a spray capillary. High voltage applied (3-6kV).

(Macfarlane and Torgerson, 1976, Zaikin and Halket, 2006, Raffaelli and Saba, 2003, Pitt, 2009, El-Aneed et al., 2009, Awad et al., 2015, Wilm and Mann, 1994, El-Faramawy et al., 2005)

APCI, Atmospheric pressure chemical ionisation; APPI, atmospheric pressure photoionisation; CI, chemical ionisation; EI, electron ionisation; ESI electrospray ionisation; FAB , fast atom bombardment; MALDI, matrix-assisted laser-desorption ionisation; PD, plasma desorption

1.5.2.1 MALDI ionisation

The term and concept of MALDI was first introduced by Karas et al in 1985 where they found that the ionisation of Alanine in the presence of Tryptophan was enhanced at a laser wavelength of 266nm. The breakthrough for large molecule laser desorption/ionisation was achieved in 1987 when Tanaka et al managed to ionise proteins with a large molecular mass of up to 34000 daltons using fine metal powder matrix dispersed in glycerol and a pulsed N_2 laser at 337nm. There were further reports from the Hillenkamp group demonstrating MALDI on larger peptides and the use of a matrix for pulsed laser desorption MS. Currently used matrices of cinnamic acid derivatives including sinapinic acids and laser wavelengths between 260nm and 360nm was suggested in 1989 (Karas et al., 1985, Karas and Hillenkamp, 1988, Beavis et al., 1989, Tanaka et al., 1988, Karas et al., 1987, Hillenkamp, 1986).

MALDI can be described as photo-volatilisation of the sample co-crystallised in a UV-absorbing matrix. The mechanism of ionisation in MALDI involves desorption and ionisation of the analyte which is co-crystallised within the matrix from the solid phase. MALDI ionisation is a two-stage process which begins with photo-ionisation of the matrix using usually a N_2 laser beam at 337nm which brings the crystallised sample from the solid phase into the gas phase. The matrix absorbs this laser energy thought to be due to energy pooling and multiphoton absorption. The second stage ionisation of the analyte from a collision process with a matrix ion by protonation or deprotonation forming positive or negative ions respectively (Figure 1.7) (Kong et al., 2001, Chang et al., 2007).

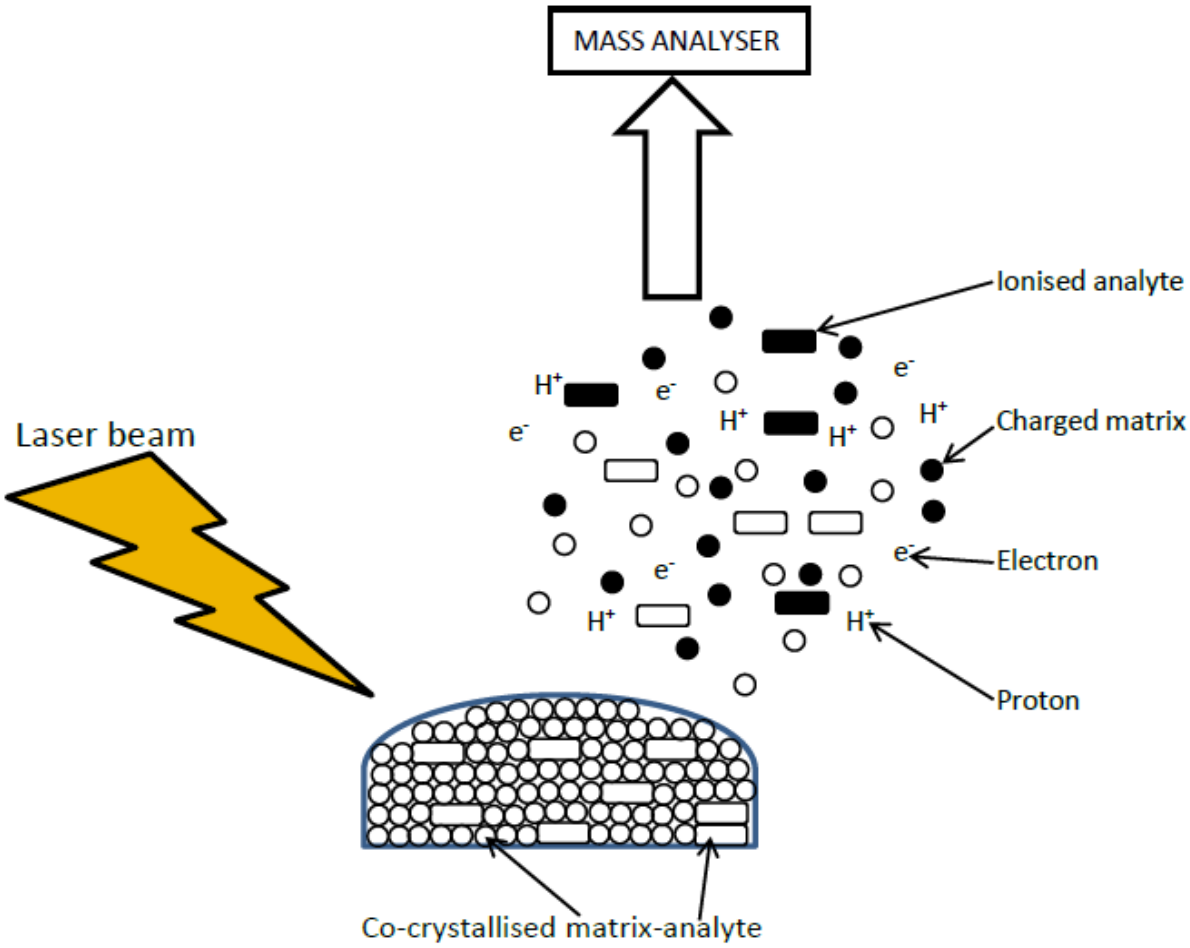


Figure 1.8 MALDI Ionisation whereby the co-crystallised matrix is bombarded by a laser beam (usually N_2 337nm). Ablation of the crystal and matrix excitation results in the charge being transferred to the analyte forming ionised analytes before proceeding towards the mass analyser.

The processes of ion generation and desorption using the MALDI technique are still poorly understood however, there are many ideas where ions can be generated involving both chemical and physical pathways which include; gas-phase protonation, ion-molecule reactions, disproportionation, excited-state photon transfer, energy pooling, thermal ionisation, and desorption of preformed ions. From this there are two main proposed models that could explain the ionisation process; gas-phase protonation and the Lucky Survivor Model (Marvin et al., 2003).

Gas Phase Protonation Model

This is a two-step model where the initial step is photoionisation of the matrix crystal forming matrix ions, and the second step is ion-molecule reactions, also known as charge transfer, whereby neutral analytes become ions by collisions between the charged matrix and neutral analyte. The model assumes that the analyte is released in a neutral form and becomes protonated by gas-phase proton transfer by a charged matrix molecule. This model can be represented by the following equation; $X + MH^+ \rightarrow XH^+ + M$ where X is the analyte, MH^+ represents the charged matrix transferring the charge over to the analyte. Land and Kinsell also supported this theory as a two photon ionisation process where proton transfer occurred between sinapinic acid and biomolecular analytes. They found that there was a correlation between charged matrix precursors (MH^+) and the intensity of protonated analytes (XH^+) (Moon et al., 2012, Ehring et al., 1992, Land and Kinsel, 1998).

Lucky Survivor Model

The assumption of the Lucky Survivor model (also known as the Cluster Model) is that the analyte occurs as a pre-formed ion inside the matrix crystal and is released following laser irradiation as a cluster of analyte ions, matrix molecules and corresponding amounts of counter-ions. Here quantitative charge neutralisation occurs where positively charged clusters (analyte ions, matrix molecules and counter-ions) undergo neutral loss releasing singly charged analyte ions. These are known as the Lucky Survivors (Moon et al., 2012).

However, there are disadvantages of the model, firstly the origin of fragment ions are highly charged ions which have been partially neutralised by electron trapping. Since analytes exist as pre-charged species, excess electrons can neutralise the pre-charged molecules. Secondly, electron neutralisation can occur where the formation of radicals impacts on the presence of background interference on the mass spectra. Due to the disadvantages a refined model was developed (Karas et al., 2000).

Refined Model

The refined model begins with the same assumption that analyte molecules are pre-formed ions within the matrix crystal. However, the reformed model shows counter-ion neutralisation occurs through excess matrix as ionised molecules isolating pre-charged analyte clusters and leading to singly charged analyte ions. In essence this model shares similarity between both the original Lucky Survivor Model and the Gas Phase Protonation Model since they both involve protonated matrix ions to form analyte ions (Karas and Krüger, 2003).

Jaskolla and Karas investigated these two proposed theories and combined the gas-phase protonation and Lucky Survivor model to show analyte protonation. It was discovered by using a deuterated matrix that numerous factors instigate which of the two protonation pathways are favoured. These factors include, but are not limited to, laser fluence, proton affinity of the choice of matrix and analyte, ratio of protonated-to-neutral analyte co-crystallised within the matrix, analyte composition and size. Findings showed that with increasing size the peptides preferred route of protonation via the Lucky Survivor Model, possibly due to increased intramolecular folding of the analyte in the matrix crystal which results in a higher chance of survival of precharged ions. With the choice of matrix, α-CHCA showed a preference for ionisation to occur via the Lucky Survivor Model, whereas by using more reactive matrices with lower proton affinities the preference for the Gas Phase Protonation Model is increased. Laser fluence increase also showed a preference for Gas Phase Protonation whereas the increase in quantitative analytes in solution and intramolecular ion stabilisation preferred the Lucky Survivor Model (Jaskolla and Karas, 2011).

Matrix

In MALDI, the choice of matrix is possibly the most critical component in ensuring that ionisation of the analyte occurs. Matrix solutions used for proteins tend to be derivatives or benzoic acid, cinnamic acid and other aromatic compounds. The most common matrices used are α-CHCA, SA, DHB and HPA (Marvin et al., 2003). It is suggested that for positive ions acidic matrices are preferred whereas for negative ions basic matrices are preferred. Previous studies have outlined three major functions of the matrix; incorporate the analyte into a co-crystallised matrix, its role in collective absorption and ablation, and its role in the soft ionisation technique that is central to minimal energy transfer to the analytes ensuring they are analysed efficiently (Glückmann et al., 2001, Baldwin, 2005).

The choice of matrix is dependent on the type of analysis. It was found that DHB is a useful matrix for proteins, peptides, DNA and synthetic polymers. α-CHCA is also a useful matrix for proteins and peptides, but not DNA since the ionic signal of DNA cannot be detected. SA was shown to be used for proteins and polymers whereas HPA was shown to be the best matrix for DNA studies (Kong et al., 2001). In proteomics, α-CHCA and DHB are commonly used and comparisons between them showed that α-CHCA has better sensitivity to detect greater number of peptides in samples with low femtomole concentrations in contrast to DHB which preferred samples with higher peptide concentrations. Also, α-CHCA was better at detecting peptides at an *m/z* range from 1500, whereas DHB showed preference at *m/z* of less than 1000. Compared to the use of single matrix solution, a matrix mixture offers a slightly improved *m/z* range coverage capitalising on the <1000 *m/z* range of DHB and >1500 *m/z* range of α-CHCA. In addition to this, a matrix mixture also offered improved detection sensitivity due to reduced chemical noise compared to single matrix use, and dramatically improved shot-to-shot reproducibility over the sample surface compared to DHB (Zhang et al., 2010, Laugesen and Roepstorff, 2003).

1.5.2.2 Electrospray ionisation

Electrospray ionisation (ESI) was first introduced in the 1960s where Dole et al demonstrated the formation of ions by electrospraying a dilute polymer solution into an evaporation chamber. ESI begins with a liquid containing the analyte passing through a capillary tube/spray needle at a rate of 1-1000µL/min under the influence of a high voltage (3-6kV) at atmospheric pressure which produces highly charged droplets which are nebulised from a needle tip into the atmosphere. The droplets are directed through the tip by a stream of sheath gas (nitrogen) around the tip during nebulisation. Remaining solvent molecules are removed by the passing of these droplets through a curtain of heated inert gas, usually N_2, or a heated capillary. Ions formed then pass through an orifice towards the mass analyser (Dole et al., 1968, Awad et al., 2015).

ESI is often used in combination with chromatography to separate the sample before ionisation especially when using non-volatile, labile, and high molecular weight compounds. To further this advancement nanospray technology dealing with low flow rate and enhancing ion formation has also been developed (Wood et al., 2006).

Despite the debate about the production of ions by solvent evaporation, two major mechanisms have been proposed for ionisation; Ion Evaporation Theory and Charge Residue Theory (Figure 1.8).

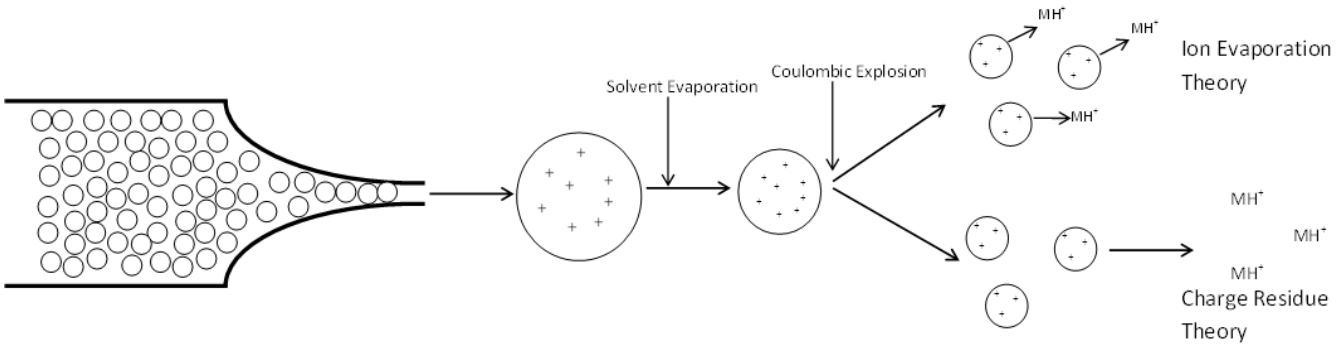


Figure 1.9 ESI Ionisation showing the two proposed ways of ionisation using ESI; Ion Evaporation theory and Charge Residue Theory. Showing the similarities between the two theories where solvent evaporation results in reduced size of the droplet until it reaches the Rayleigh Limit whereby Coulombic Explosion occurs. From here the differences between the theories are displayed.

1.5.3 Mass analyser

Following ionisation, ions are accelerated towards and driven through the mass analyser by either an electric or magnetic field where the ions are separated according to their *m/z*. Numerous mass analysers are available and vary according to price, size, mass range, analysis speed, sensitivity, resolution and ion transmission. Common mass analysers include time of flight (ToF) and quadrupole which are ion free flight and scanning analysers respectively, and ion trap, FT-ICR and Orbitrap which are trapping analysers. Hybrid analysers are also available such as Q-ToF (quadrupole attached to ToF instrument) to exploit the scanning capabilities of the quadrupole and the resolving power of the ToF (Yates et al., 2009).

1.5.3.1 Time of flight

Time of fight, as the name suggests, separates ions according to the time they take to fly through a tube before reaching the detector. The linear ToF is composed of two sections; an ion acceleration region which contains an ion-optic assembly and a field-free flight which contains an ion drift tube. The ion acceleration region begins with ions formed at the same time and location but differ in molecular weight. Here a voltage potential is applied and an electric field created between two elements contained within the ion-optic assembly allowing the ions to be accelerated through one of the elements and into the drift tube before reaching the detector. This can be portrayed by the equation $m/z = K\ t^2_f$ where K is the calibrating factor and t_f is the time of flight (Merchant and Weinberger, 2000).

Advantages of the ToF include; the ability to record a mass spectrum in a few microseconds, a large mass range for the ions, the ability to display the full mass spectrum as well as individual parts of it, and due to the field-free flight all of the formed ions will eventually reach the detector. The main disadvantage of the ToF is the poor resolution due to the ions in the ToF acquiring different initial velocities. This was overcome with the use of a reflectron where the path of the ions is altered. Ions travelling at a higher velocity will travel deeper into the reflectron and repelled into a longer ion drift tube thereby improving resolution (Mamyrin et al., 1973).

The reflectron allowed an unlimited mass range up to a few million Dalton, higher resolution compared to the linear ToF due to the longer free-flight path, noise suppression, and improved mass measurement accuracy due to the longer ion flight time. ToF is commonly used with a MALDI ion source; however use with ESI in hybrid instruments can also be applied (Figure 1.9) (Mamyrin, 2001).

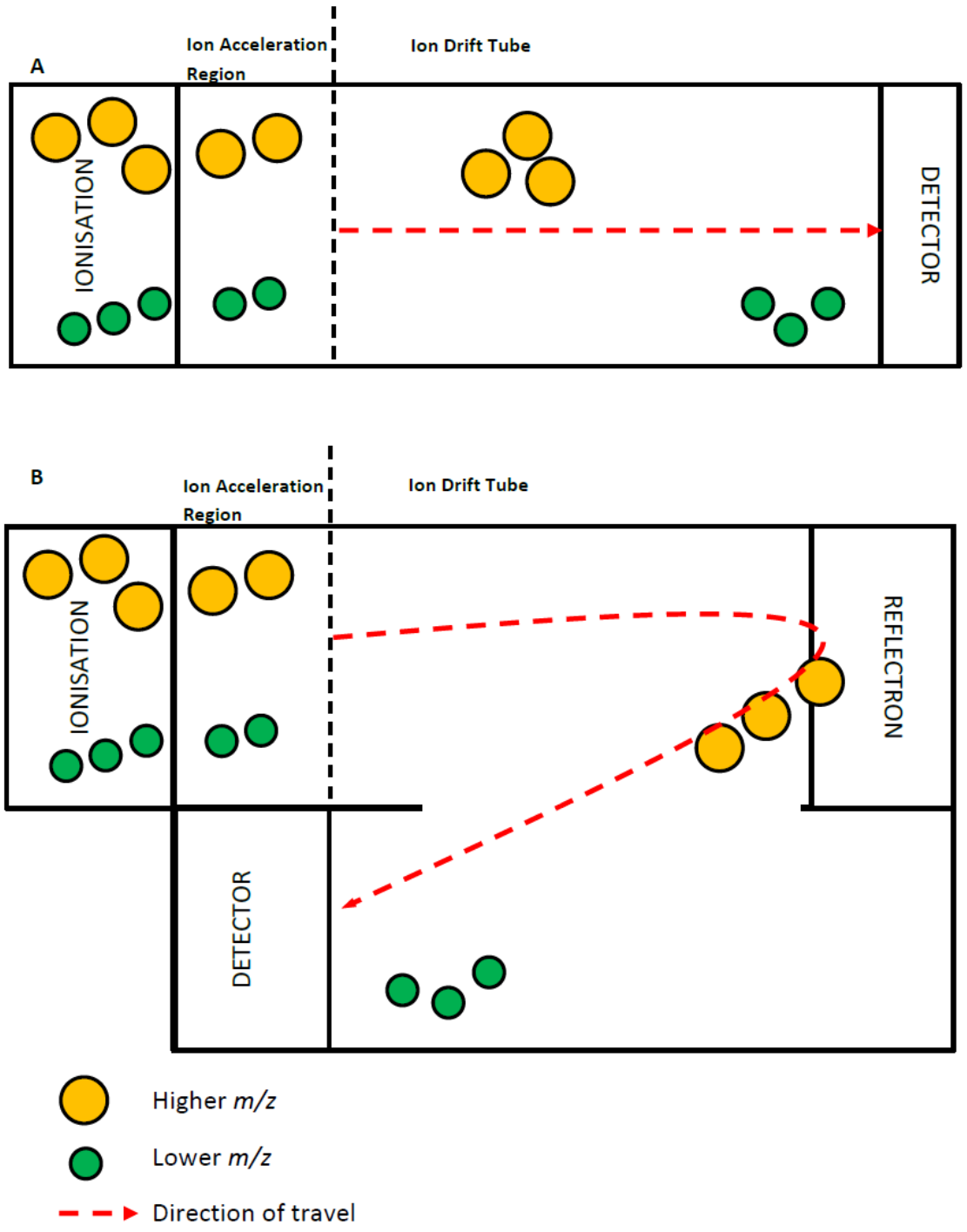


Figure 1.10 Depiction of time of flight mass analysers; (A) Linear ToF and (B) Reflectron ToF

1.5.3.2 Quadrupole

The quadrupole consists of four parallel metal rods that are accurately positioned in a radial array. Opposite and diagonal rods are connected electrically with direct current (DC) and radio frequency is applied to the other two causing ions to travel in the *z* direction with oscillations in the *x*-*y* plane. The oscillations applied can be controlled by altering the voltages applied to the rods. Positively charged ions will move towards the negatively charged rod. Ions travelling with the chosen *m/z* and correct trajectory will reach the detector without hitting any of the rods. Whereas the remaining ions will hit the metal rods, become neutralised, without reaching the detector (Figure 1.10). The main advantages of the quadrupole include its low cost and maintenance, robustness and small size making it useful for a variety of applications. However, only ions with a small *m/z* range are allowed to pass through (Ho et al., 2003, Miller and Denton, 1986).

The problem associated with the quadrupole of a small mass range can be overcome by linking it to other quadrupoles (triple quadrupole) or creating a hybrid by attaching it to a ToF thereby creating the Q-ToF.

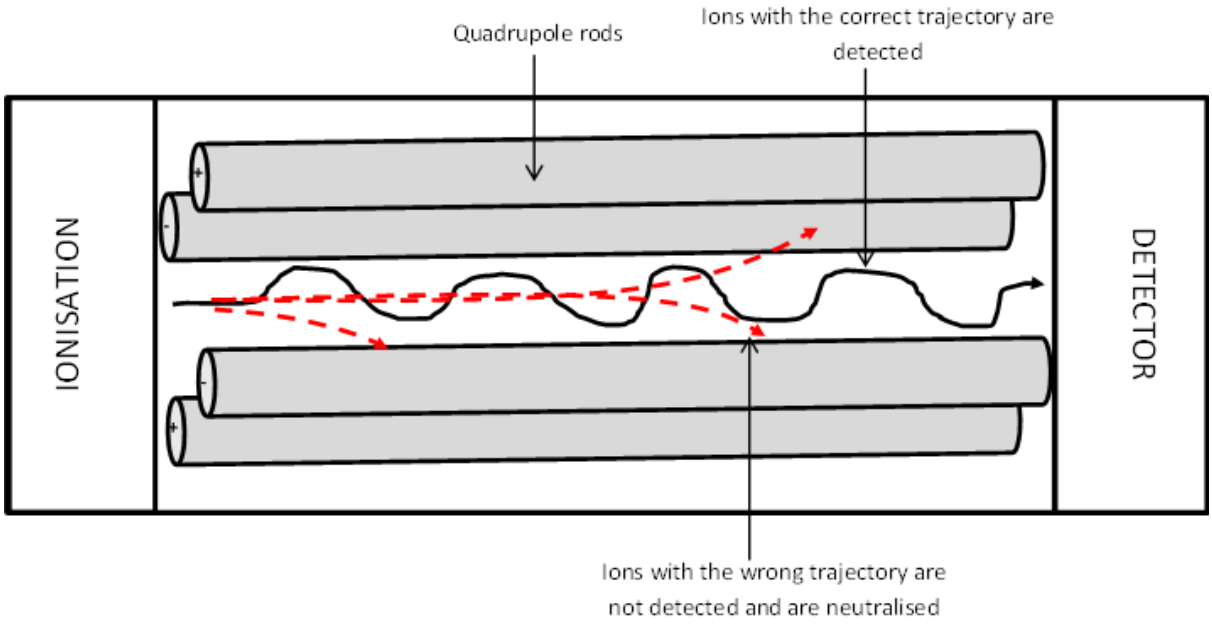


Figure 1.11 Quadrupole mass analyser: Showing the arrangement of the four metal rods positioned in a radial array with opposite and diagonal rods connected to either AC or DC causing oscillations in the *x*-*y* plane of ions which are then either neutralised or detected.

1.5.3.3 Triple quadrupole

A triple quadrupole (QqQ) MS is a tandem mass spectrometer consisting of two quadrupole mass filters (termed Q1 and Q3) in series separated by a RF-only quadrupole (Q2) where a collision gas can be introduced for collision induced dissociation (CID) to occur to further fragment ions. In single ion mode (MS), the collision gas is turned off and ions are resolved at either Q1 or Q3 the remaining quadrupole (Q1 or Q3) operated in total ion mode. When operated as MS/MS, Q1 is used to select precursor ions and Q3 is used to provide mass analysis of the molecular ions. Comparing the operation of MS and MS/MS, the latter results in less sensitivity whereas the gain in signal-to-noise due to the reduction in background interference and the additional specificity offered by double mass analysis, merits in improved detection limits and quantitation than MS (Thomson et al., 1995).

Depending on the mode of operation and the required output of information, MS/MS allows several modes to be employed. The simplest mode is the 'product ion scan' where a precursor ion is selected by *m/z* at Q1. This ion then fragments at Q2 and its products are analysed at Q3, providing structural information. The second mode of operation is the 'precursor ion scan' which focusses Q3 on a selected ion and utilises Q1 to scan the masses. This results in many ions identified at Q1 that produce the selected mass (at Q3) by fragmentation are detected. The third common operation is the 'neutral loss scan' where both analysers (Q1 and Q3) scan together but with a constant mass offset between the two. The mass difference corresponds to a neutral fragment lost from an ion in Q2 (Hopfgartner et al., 2004, Chernushevich et al., 2001, Domon and Aebersold, 2006).

MS/MS involving QqQ as the mass analyser source, various modes can be applied for targeted proteomics, however typically the SRM (Selected Reaction Monitoring) mode is used. This allows quantitation of data by targeting a set of peptides depending on SRM transitions where Q1 and Q3 are filters used to select out certain *m/z.* For this to occur, Q1 is used to select the precursor peptide ion of interest *(m/z*), which is then fragmented by CID in Q2 to yield product ions. From the resulting product ions, selected fragment (s) are analysed for quantitative analyses (Lange et al., 2008, Hird et al., 2014).

1.5.3.4 Other mass analysers

Other mass analysers include MS/MS, hybrid Q-ToF, ion trap and Fourier transform ion cyclotron resonance (FT-ICR). MS/MS includes an additional mass analyser such as a quadrupole and ToF (Q-ToF) or a triple quadrupole (QqQ, the most commonly used tandem MS) separated by a collision chamber(s). The first mass analyser is involved in precursor ion selection followed by a collision cell where ions are dissociated producing fragment ions before entering the second mass analyser where ions are separated and selected for progression towards the detector. The collision cell in the triple quadrupole is the second quadrupole. MS/MS is often used in proteomics for protein identification where it can selectively isolate a protein of interest (de Hoffmann, 1996).

Hybrid Q-ToF combines the scanning powers of the quadrupole and the resolution of the ToF. Q-ToF models consist of two quadrupoles, the latter being a collision cell, attached to a high pressure collision cell to a ToF in an orthogonal fashion. The Q-ToF can also be interfaced with ESI or MALDI, MS/MS and with liquid chromatography as a separation technique (Glish and Burinsky, 2008, Morris et al., 1996).

The Orbitrap is a newly developed technique based on trapping ions in an electrostatic field around a central spindle. The ions trapped begin to oscillate harmonically along the axis of the applied electric field. The frequency of the oscillations is relative to the m/z. A mass spectrum is produced when the ion frequencies are measured of the image current in the outer electrode and with the Fourier transforms used to create a spectrum. The Orbitrap has shown to have high mass resolutions, high mass accuracy and a high m/z range (Hu et al., 2005, Han et al., 2008).

Similar to the Orbitrap, the FT-ICR is a technique based on image current detection; however it used detection from excited ion cyclotron motion where *m/z* is inversely proportional to the cyclotron frequency. The ions are trapped by an electric field, along the magnetic field vector and orthogonal to the magnetic field, in a measuring cell (Penning trap). The ions are excited in the cyclotron motion after RF frequency is applied. The charges from these ions are detected by electrodes and the current signal recorded and transformed into a mass spectrum. FT-ICR offers ultrahigh resolving

power and mass accuracy compared to other mass analysers (Marshall and Hendrickson, 2002, Nikolaev et al., 2014).

1.5.4 Detector

The final part of mass spectrometry is the detector. Ions travelling towards the detector carry an electrical charge which can be amplified and recorded as m/z. Different detector applications are used to suit the needs of the sample of analysis, the type of ionisation technique used, the type of mass analyser employed and the programming of the instrument. Types of detectors include electron multiplier, microchannel plate, inductive detector, photon emission, charged induced potentials and the Faraday cup (Baldwin, 2005, Chernushevich et al., 2001, Park et al., 1994, Bahr et al., 1996).

1.5.5 Separation techniques

Due to the complexity of proteins, separation techniques are often applied and used in conjunction with MS and involve various gel-free and gel-based techniques. The most commonly used gel-free techniques commonly involve chromatography techniques include gas chromatography, liquid chromatography, and capillary electrophoresis MS and ion mobility. These techniques often require specialised equipment and cost more than gel-based techniques. They are easily coupled to mass spectrometers, as in LC-MS, offer increased sample recovery, reproducibility and higher throughput. Gel-based techniques include SDS-PAGE and gel liquid chromatography MS/MS. Gel-based techniques are cheap, simple and easy to perform removing contaminants and fractionating the sample (Jafari et al., 2012).

1.5.5.1 Liquid chromatography

Liquid chromatography is an analytical technique used to separate proteins or biological material of interest by using two phases: mobile phase and stationary phase. LC involves a liquid mobile phase with the flow of liquid is maintained by a pump. In the mobile phase the sample is injected through a column, known as the stationary phase, containing densely packed material. In the stationary phase, proteins of interest with complementary binding sites with the affinity-bound ligand are captured. The captured protein is then eluted by changing the conditions of the LC system. Selecting a group of

proteins can be achieved by altering the conditions in the mobile phase (buffer pH, salt concentration and pH) or stationary phase (type of column, ligand, antibodies, size exclusion and gel-filled) (Issaq, 2001).

HPLC, in contrast to LC, operates with a higher pressure pump (1000-3000psi) ensuring the separation of proteins is performed with a greater resolution compared to LC. The higher pressure results in smaller column diameter and particle size, however this LC method is limited to pressure (Jerkovich et al., 2003). Ultra-high-performance liquid chromatography (UHPLC) is a new version of HPLC and can typically involve reduced particle diameter (2µm) and at higher pressures compared to HPLC (6000-15000psi). Its main advantages include resolution, speed and sensitivity where similar results can be achieved as HPLC but in one tenth of the time (Plumb et al., 2004). Reversed-phase chromatography (RP-LC) is the most commonly used chromatography method and involves separation based on hydrophobicity which is achieved by employing a polar mobile phase and (hydrophobic) non-polar stationary phase. Due to the interaction between the hydrophobic stationary phase and a polar mobile phase has led to RP-LC being the most widely used separation technique in proteomics. Other chromatography techniques include nano-LC using flow rates in the nanoliter/minute range, ion-exchange chromatography involving the separation of proteins according to their electric charge, and hydrophilic interaction (HILIC) where hydrophilic peptides are separated (Manadas et al., 2010).

One of the problems with mass spectrometry ionisation is that matrix suppression can affect ESI resulting in the analyte formation rate and signal response differs. Separation techniques are employed to prevent the matrix from interfering with the analyte. LC-MS/MS approach was taken to analyse the effects and results showed that using LC-LC (also referred to as 2-D LC) by operating two separation techniques rather than LC-MS or LC-MS/MS can be used to compensate for matrix suppression (Choi et al., 2001).

Advantages of LC based separation techniques include increased sensitivity, specificity and better reproducibility compared to directly injected methods with MS. LC-MS also allows the analysis of multiple analytes with a single run and can be used with numerous mass analysers including quadrupole, QqQ, Q-ToF and ion traps (Pitt, 2009).

1.5.6 Conclusion

Proteomics is an emerging field of research since the completion of the human genome project. Proteins form an abundant part of the proteome providing crucial biological information at a certain time point and often related to a disease or disease severity.

MS based proteomics has been shown to be indispensable in biological and medical research. It allows high throughput of data with great sensitivity and specificity using upcoming technology often coupled to various instruments to provide novel information that would otherwise be difficult to obtain. Biomarkers are important in the diagnosis of many diseases are often detected and discovered using an MS based approach.

1.6 Current clinical biomarkers and clinical need

1.6.1 Biomarker characteristics

The term 'biomarker' was defined by the National Institute of Health Consortium as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Group et al., 2001). This was further developed by the American Heart Association for evaluating and standardising newer biomarkers for clinical use in CVD such that a novel biomarker should be evaluated in phases including proof of concept, validation in independent populations, added value of biomarker when added to standard risk markers, effects on patient management and outcomes, and cost effectiveness (Hlatky et al., 2009).

A biomarker can be measured in samples such as; plasma, serum, urine or tissue, or they may form a part of an imaging test such as CT or MRI. From a precision medicine approach they are able to indicate health and disease characteristics and may serve as response to treatment, indicators of disease (diagnostic), disease state (clinical or preclinical), rate of disease, screening, staging or prognostic markers to predict course of disease such as mortality (Vasan, 2006).

Important characteristics of an ideal biomarker assay include; accuracy, precision, sensitivity and specificity. In addition to this it is essential that reference limits by comparing with a healthy population and limits of detection are established showing the levels of reproducibility over time. In disease, a biomarker should further show clinical information regarding the disease such as BNP concentrations increasing with worsening HF. Biomarkers should be validated in different cohorts (Dhingra and Vasan, 2017).

1.6.2 Current clinical biomarkers and proteomics

Molecular biology tools have enabled for further biomarker discoveries including those from genomics and genetic studies, molecular imaging metabolomics and proteomics. Proteomics is an ever-expanding field that uses techniques such as mass spectrometry to measure proteins and peptides to identify novel markers. It serves as an effective

medium for novel discoveries since in excess of 20,000 protein coding genes are responsible for over 1 million protein found in biological matrices (Jensen, 2004). Its current applications, commonly in plasma, serum, urine, saliva, and tissue samples, (Legrain et al., 2011) has provided critical advancements in clinical science through the development of diagnostic and prognostic assays for patients presenting with, or at risk of, a multitude of diseases (Chan and Ng, 2010).

The use of proteomic approaches and measurement has been particularly beneficial for the assessment of CVD, with the notable inclusion of natriuretic peptides and troponin isoforms in clinical decision making for HF (Maisel, 2002) and acute coronary syndromes, (Newby et al., 1998) respectively. There are many CVD biomarkers that are currently available and have clinical use as diagnostic, prognostic or predictive markers, in particular, biomarkers for HF and MI will be further discussed. Frequently used biomarkers (BNP/NT-proBNP and troponin) are discussed and other novel associated biomarkers briefed according to their various pathophysiological processes.

1.6.2.1 BNP/NT-proBNP

BNP is perhaps the most widely used biomarker for cardiac stress. It is central to cardiovascular homeostasis and released from the cardiomyocytes, primarily located in the ventricles, in response to stress and stretch of the cardiac muscle (Levin et al., 1998). Upon binding to specific receptors, BNP is activated and initiates a reduction in systemic vascular resistance, inhibits the actions of the renin-angiotensin-aldosterone system (RAAS), and promotes vasodilation and natriuresis (de Sa and Chen, 2008). BNP has been studied extensively for its role as a diagnostic (Dao et al., 2001, McCullough et al., 2002) and prognostic (Jourdain et al., 2007, Cheng et al., 2001) biomarker in HF, including both chronic patients and acute decompensated admissions. However, an important limitation of BNP for HF diagnosis is that circulating levels may become elevated in response to alternative disorders such as renal dysfunction, left ventricular hypertrophy and right ventricular dysfunction (Luchner et al., 2000). Furthermore, as factors such as sex, age and body mass index are also associated with fluctuations in BNP levels, accurate interpretation of circulating concentrations is crucial (Maisel et al., 2008).

Circulating levels of natriuretic peptides are low in healthy individuals. They are released from the cardiomyocytes in response to stress, stretch and volume overload as in HF, and are therefore commonly used and recommended by NICE and ESC guidelines. Two common circulating forms exists and are measured clinically; NT-proBNP and BNP. The levels of both natriuretic peptides are increased in HF and show a strong correlation with severity of HF and myocardial stretch (Kim and Januzzi, 2011). Suggestive cut-offs to rule out HF using BNP is 100 pg/mL and 300 pg/mL for NT-proBNP. The sensitivities for both peptides range from 90-99% however the specificity is more within the range of 75% (Roberts et al., 2015). However, natriuretic peptides levels are also influenced and increased by other factors such as age, gender (female) and renal function.

In addition to uses in diagnosis and prognosis, its role in monitoring treatment with diuretics and vasodilators including ACE inhibitors (Felker et al., 2009), angiotensin-II receptor antagonists (Ewald et al., 2008), and aldosterone inhibitors (Januzzi et al., 2006) has also shown promise. Circulating BNP levels are known to fall rapidly following successful treatment strategies, therefore repeat measurements of BNP concentrations provide an observation of responses to medical interventions.

Although widely used in clinical analysis, BNP exhibits a short half-life (approximately 20 minutes) when present in the circulation (Potter, 2011) and, therefore, care must be taken during the sampling and storing of blood samples. N-terminal proBNP (NT-proBNP) is released in conjunction with BNP and is considered as a more stable alternative due to its longer half-life (Mueller et al., 2004). NT-proBNP has shown comparable qualities to BNP as a biomarker for diagnosis (McDonagh et al., 2004), diagnosis in primary care with suspected HF (Gustafsson et al., 2005) and, for HF hospitalisation in emergency (Ozturk et al., 2011). Comparable qualities have also been demonstrated for prognosis (Taylor et al., 2014, Ewald et al., 2008), prognosis in primary care (Gustafsson et al., 2005), and guided treatment (Lainchbury et al., 2009) in HF.

When studied in direct comparison, BNP and NT-proBNP show comparable utility for diagnosis (Ewald et al., 2008), prognosis (Seino et al., 2004, Bettencourt, 2004) and biomarker-guided therapy (Roberts et al., 2015) in chronic HF, with reductions in all-cause mortality reported with titration of therapies based on repeat measurements.

Circulating levels of these natriuretic peptide biomarkers are increased in HF and are strongly associated with disease severity and myocardial stretch (Kim and Januzzi, 2011). Studies, such as the Valsartan Heart Failure Trial (Val-HeFT), have also shown BNP and NT-proBNP to provide superior prognostic information when compared to alternative neurohormonal markers of risk (Masson et al., 2006).

In addition to this, as biomarkers in MI, both BNP and NT-proBNP are used in prognosis. Elevated levels of BNP or NT-proBNP have been shown to be associated with in-hospital (Scirica et al., 2013) and short-term (30-day) mortality (Mega et al., 2004), as well as mortality at extended follow-up periods of 4 years or more (Omland et al., 1996, Richards et al., 2003, Omland et al., 2002). Circulating levels of BNP have also shown relationships with infarct size (Mayr et al., 2011), systolic dysfunction (Gunes et al., 2008), development towards heart failure (Darbar et al., 1996) and cardiac death (Fazlinezhad et al., 2011). Even beyond the impact of common MI markers such as troponin and copeptin, has BNP provided additional information (Omland et al., 1996).

1.6.2.2 Troponin

Troponin proteins are found in cardiac and skeletal muscle tissue and are involved in the regulation of actin and myosin interactions during muscle contraction. Troponin I (cTnI) and T (cTnT) are unique and exist only in cardiac muscle allowing the measurement of these specific isoforms to provide information in cardiovascular disease, notably as diagnostic biomarkers for ACS . However, development of high-sensitivity troponin assays have further allowed the measurement of elevated cTnI/cTnT levels in HF patients (Shah et al., 2018), with increased concentrations associated with poor outcome. High-sensitivity assays for cTnT have been applied for prognosis in acute HF, with circulating concentrations associated with disease severity and worse outcomes particularly for 180-day cardiovascular mortality (Felker et al., 2015). Furthermore, cTnT has been shown as a suitable marker to reflect myocardial damage and cardiac dysfunction in HF , associated with long-term mortality and HF hospitalisation adding to the ability of BNP (Shah et al., 2018), and showed prognostic information in older patients (>60 years) with chronic ischaemic HF (Gravning et al., 2013). For new diagnosis of HF, post-MI, the Long-Term Intervention with Pravastatin

in Ischemic Disease (LIPID) study found patients with increased troponin (cTnI) levels from baseline to 1 year to be associated with HF hospitalisation (White et al., 2014).

In addition, high sensitivity cTnI has been shown to reflect elevated NT-proBNP levels, and reflect as an independent and useful prognostic predictor in chronic HF patients (Tsutamoto et al., 2010). For acute admissions, serial changes in cTnI levels over 90 days were functional in predicting increased likelihood of mortality and rehospitalisation (Xue et al., 2011). When used in combination with BNP measurements, cTnI measurements on admission were shown to predict in-hospital mortality, and increasing concentrations were associated with risk of death in a large-scale registry cohort (The Acute Decompensated Heart Failure National Registry (ADHERE)) (Fonarow et al., 2008b, Peacock IV et al., 2008).

For diagnosis of MI, troponin replaced CK-MB as the biomarker for choice in 2000 (Antman et al., 2000). Troponin is released in response to irreversible myocardial damage and is highly specific to cardiac tissue making it suitable in CVD . As a prognostic marker, its level correlates with the size of infarct and thus give clinicians prognostic information. The early detection of MI can become complicated due to troponin kinetics, however high-sensitivity troponin assays are thought to overcome this. They have shown higher levels of accuracy for the early diagnosis of MI and have shown nearly absolute specificity and high clinical sensitivity for myocardial necrosis. The sensitivity and specificity for high-sensitive cTnT levels >99th percentile in AMI has shown to be 95% and 80% respectively, with 72% and 97% respectively for standard cTnT levels showing the increased sensitivity comes at a cost with the specificity (Xu et al., 2013). Even as prognostic markers, elevated troponin levels have demonstrated worse outcomes. The MISSION! Trial, for example, has shown cTnT levels reflect size of infarct and are able to predict left ventricular function at 3 months, and major adverse cardiac events at 1 year (Hassan et al., 2009). For cTnI, the TIMI-IIIB trial showed that elevated cTnI at admission was related to increasing mortality in ACS patients (Antman et al., 1996). Troponin remains an important and widely used marker for diagnosis and prognosis in ACS, yet further studies are required to determine the added ability in the prognosis of high-sensitivity troponin assays (Daubert and Jeremias, 2010).

1.6.2.3 Other markers

Other markers, some of which are newer, have shown promise in HF and MI are shown in Figure 1.11. Some common ones are discussed further.

Markers such as ANP have been identified for a role in CVD even before BNP. However despite showing prognostic utility in HF, results have shown that due to lower levels of stability in circulation, ANP is inferior as a biomarker to BNP. Molecular forms of ANP, such as N-terminal ANP (N-ANP/NT-proANP) and mid-regional pro-ANP (MRproANP), have also been shown to offer diagnostic and prognostic roles in HF due to them displaying greater stability (Luers et al., 2013, Lerman et al., 1993). The Biomarkers in ACute Heart Failure (BACH) cohort reported that MRproANP was a suitable diagnostic and prognostic biomarker in dyspneic patients, with results comparable to BNP (Maisel et al., 2010).

C-reactive protein (CRP), is a traditional marker that has been extensively applied to other conditions such as arthritis in its role as a marker of inflammation. Elevated levels are associated with mortality in acute MI and also increased CRP levels in HF patients, reflecting myocardial damage (Berton et al., 2003) and associations with HF severity, mortality and morbidity (Anand et al., 2005), and rehospitalisation (Alonso-Martinez et al., 2002).

Whilst BNP is an example of a biomarker that was discovered from targeted physiologic studies, the discovery of ST2 shows how novel markers can be discovered using platform including proteomics, genomics and metabolomics employing an 'unbiased' or non-targeted approach. Primarily employing genomics, ST2 gene expression was induced in response to mechanical strain of cultured cardiomyocytes leading to its discovery (Gibbons et al., 2004). ST2, a member of the interleukin receptor (IL-1) family, basally expressed by cardiomyocytes and is detectable in circulation in its soluble form and found to be elevated in response to mechanical stress of the heart (Weinberg et al., 2003). Its role has been recognised in HF as an independent predictor of mortality or need for transplantation in severe chronic HF patients (NYHA class III/IV) (Weinberg et al., 2003), as well as providing prognostic information for acute HF patients when combined with natriuretic peptides (Rehman et al., 2008), suggesting its use as a

biomarker in combination with current clinical testing strategies (e.g. BNP and NT-proBNP). Furthermore, the PRIDE (Pro-Brain Natriuretic Peptide Investigation of Dyspnea in the Emergency Department) study showed that ST2 was a suitable biomarker to predict 1-year mortality in dyspneic patients, irrespective of a positive or negative diagnosis of acute destabilizing HF (Januzzi et al., 2007), offering a more generalised biomarker function that can be further refined when applying a multi-biomarker approach.

Copeptin has notable clinical interest as a biomarker in CVD, not just for HF and MI. it is the results of pre-pro-vasopressin cleavages, forming copeptin, neurophysin II and vasopressin, with the latter also known as antidiuretic hormone that is involved in fluid homeostasis and shown to be related to the severity of HF (Nakamura et al., 2006). However, whilst vasopressin is instable in circulation, copeptin is considered to have a high stability and is released in equimolar concentrations to vasopressin, allowing a more reliable and reproducible alternative for indirect measurement of vasopressin (Morgenthaler et al., 2006). Research into the clinical role of copeptin as a prognostic biomarker has shown comparable results to natriuretic peptides and initial indications suggest it offers a superiority in prediction for 14- and 90-day mortality in acute admissions (Frank Peacock et al., 2011, Maisel et al., 2011) and longer-term prediction at 24 months for patients across various stages of disease (Neuhold et al., 2008), as well as for those with advanced HF (Stoiser et al., 2006). Although a relatively contemporary biomarker for HF, data highlight copeptin measurements as a potential clinical tool for risk-stratification, particularly in acute cases. Due to its early release in response to neurohormonal activation, copeptin has been shown to rule-out early MI when used in addition to a negative cTnT test (Reichlin et al., 2009).

Many CVD states especially HF are associated with other co-morbidities and organ systems in particular the renal system and the respiratory system. A promising marker to emerge from this is Cystatin C. This small protein molecule that is involved in the extracellular inhibition of cathepsins and has been used as a biomarker of kidney function (Jernberg et al., 2004). It is removed from circulation through the kidneys, thus providing biomarker information for renal dysfunction and therefore an interest within CVD including MI and HF (Angelidis et al., 2013). Cystatin C has shown prognostic

capabilities in chronic HF patients as well as those with reduced ejection fraction (Huerta et al., 2016, Carrasco-Sánchez et al., 2011). Some studies have found cystatin C to be a better estimation of renal function than serum creatinine or creatinine-based GFR equations (Hermida and Tutor, 2006). These positive relationships with adverse outcome in CVD, as well as providing information on dysfunction in the renal system, signify cystatin C as a useful biomarker for a CVD and its comorbidities.

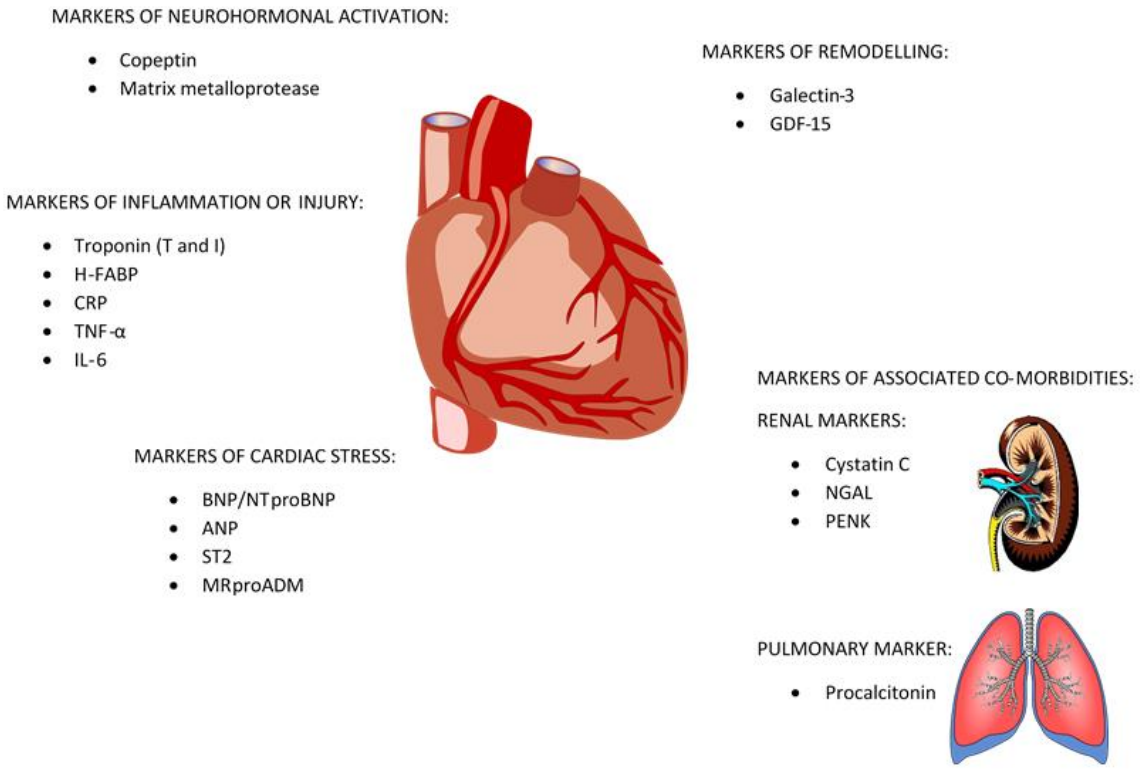


Figure 1.12 Protein biomarkers of HF and MI and their various pathophysiologic associations. ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CRP, C-reactive protein; GDF-15, growth differentiation factor-15; HFABP, heart-type fatty acid–binding protein; IL-6, interleukin-6; MRproADM, midregional proadrenomedullin; NGAL, neutrophil gelatinase-associated lipocalin; NT-proBNP, N-terminal pro–B-type natriuretic peptide; PENK, proenkephalin; TNF-a, tumor necrosis factor-a.

1.6.3 Challenges and clinical need

In clinical medicine, numerous markers have been discovered particularly in acute diagnosis of MI and HF, however the introduction into clinical practice is minimal and hampered by the costs of validation and commercialisation. The US Food and Drug Administration (FDA) approves and regulates of novel biomarkers, and has also presented challenges associated with biomarker development. The US FDA identifies a need for transparency on biomarker validation, ensure safety, effectiveness, robustness and reproducibility in its established form, and the development of approaches to overcome ethical or economical strains (Scherf et al., 2010). The biomarker pipeline is composed of processes including discovery, qualification, verification, validation and commercialisation. The reason behind the failure of novel biomarker approval by the US FDA is thought to be due to this long path from discovery to clinical assay approval (Anderson, 2005). Despite the complexities associated with the pipeline and challenges outlined by the US FDA, there are challenges associated before biomarker translation into clinics. Some of the considerations to be overcome potential challenges for biomarker discovery include sample size, inclusion/exclusion criteria, collection and handling of samples, sample processing, limitations of analytical techniques, statistical analysis, and validation in independent cohorts.

Candidate proteomic biomarkers for CVD have been discovered and research is still active. The transition of medicine towards a personalised medicine approach extends the single biomarker approach with further proteome-wide investigations have provided insight into multi-biomarker models to predict future disease developments. Hollander and colleagues have shown a promising example in this and identified 17 candidate protein biomarkers that, when combined with BNP measurements, were able to provide 97% sensitivity and 100% specificity for classifying patients on recovery from cardiac transplants (Hollander et al., 2014). This presents an opportunity for outpatient screening and monitoring response to HF treatments. There remains a need to discover and validate new biomarkers in CVD to tailor treatment and apply personalised medicine. In addition to this, applications of proteomic methods to identify markers such as Proenkephalin A (PENK) has shown utility as a prognostic marker for hospitalisation or mortality in HF and MI, and efforts to validate this for use in a clinical

setting is underway (Ng et al., 2014, Ng et al., 2017). Organisations such as Human Proteome Organisation (HUPO) have worldwide ventures under the name The Human Proteome Project (HPP), which is working to map the entire human proteome to further understanding of the localized and systems biology of proteins and protein-protein interactions for diagnostic, prognostic, and therapeutic roles in disease (Legrain et al., 2011). Efforts from discovery to validation for novel biomarkers to aid early diagnosis and prognosis in precision and personalised medicine continue.

1.7 Hypothesis, aims and objectives

Overall Hypothesis

BNP molecular forms are detectable in cardiovascular cohorts using mass spectrometry and show clinical importance and prognostic ability comparable to current clinical biomarkers, BNP and NT-proBNP.

Overall Aims and Objectives

- To develop and adapt a MALDI-ToF-MS based method to detect BNP molecular forms in cardiovascular disease cohorts; acute HF, chronic HF and acute MI.
- Perform statistical analyses to identify the clinical importance and significance for these molecular forms as biomarkers for prognosis and risk stratification.
- Develop a method that allows for high throughput and quantitation of BNP molecular forms.

CHAPTER TWO

METHOD DEVELOPMENT

2. METHOD DEVELOPMENT

2.1 Introduction

Developing a method which can be successfully used to detect BNP molecular forms in patient plasma below the cut off value for heart failure (cut off = 100pg/mL) involved starting at a previously published protocol and adapting it using locally sourced products to suit the needs of this study by taking a step-by-step approach (Fujimoto et al., 2013).

Mass spectrometry immunoassay (MS-IA) involves a two-step approach; immunoassay, whereby circulating molecular forms of BNP are captured by anti-BNP monoclonal antibodies attached to magnetic beads, and mass spectrometry where analysis is performed using MALDI-ToF MS on the captured BNP molecular forms following elution from the beads as shown in Figure 1.

Figure 2.1 Showing the steps taken to isolate the BNP molecular forms. Immunocapture was applied using magnetic beads bound to sheep-anti mouse IgG, incubated with anti-BNP antibodies, specific to the ring region of the BNP molecular forms. After elution this is then spotted onto a MALDI target plate.

Parameters experimented were; incubation time, choice of anti-BNP monoclonal antibody, ACTH concentration, lowest detection limit of synthetic BNP molecular forms, BNP detection in healthy human plasma, and detection of BNP molecular forms in spiked healthy plasma.

2.2 Mass Spectrometry Immunoassay Method Development

2.2.1 Incubation time

To analyse the effect of incubation time required to form the antigen-antibody-magnetic beads complex; BNP molecular forms were spiked into LC-MS grade water (positive control) and also into iPSC-derived cardiomyocytes, prior to MALDI-ToF-MS analysis. Incubation times of 60, 90 and 120 minutes were used in this investigation, and results are shown in Table 2.1.

ACTH at a concentration of 1 fmol/L was used as a standard for expressing the ratio of BNP molecular forms.

Table 2.1 Effect of incubation time on the signal intensity ratio of synthetic BNP molecular forms (/ACTH) as spiked into LC-MS grade water (positive control) and also in iPSC-derived cardiomyocytes

	Incubation Time (hours)	Positive control			iPSC-derived cardiomyocyte		
		BNP 5-32	BNP 4-32	BNP 3-32	BNP 5-32	BNP 4-32	BNP 3-32
Signal Intensity (/ACTH)	1	2.90	1.30	1.18	2.77	1.43	2.48
	1.5	2.38	1.28	0.95	2.55	1.52	2.62
	2	3.12	1.52	1.22	2.74	1.62	2.49

The signal intensity (/ACTH) showed that the difference between the incubation times in both the positive control and iPSC-derived cardiomyocytes for all three molecular forms was negligible. The difference between the signal intensities was not significantly increased over the course of the extra hour incubation. Therefore a 1 hour incubation time was deemed acceptable.

2.2.2 Anti-BNP antibody

To analyse the best anti-BNP antibody, the judgement criteria were based on the detection of BNP molecular forms as spiked into LC-MS grade water (positive control) and also in iPSC-derived cardiomyocytes, minimal background interference and non-specific binding. Ky-hBNP II (Shionogi), Sekisui 0 (33240), Sekisui 2 (33242) and Sekisui 7 (33247) (Sekisui Medical Co.) were used as anti-BNP antibodies. Table 2.2 shows the results.

ACTH at a concentration of 1 fmol/L was used as a standard for expressing the ratio of BNP molecular forms.

The commercially available Shionogi KYhBNP-II anti-BNP antibody is raised against the ring structure of BNP (amino acids 14–21). It detects antigens of BNP and BNP molecular forms, and possibly cross-reacts with proBNP. No information is available for the Sekisui anti-BNP antibodies (33240, 33242, 33247). It can be assumed that these antibodies are either raised against the ring structure of BNP or the COOH terminus based on the detection antigens found in this thesis (BNP molecular forms) all possessing different NH_2 amino acids. Based on this assumption, cross-reactivity with proBNP is also possible. These antibodies were kindly provided by Sekisui Medical Co. and are not commercially available. No information regarding antibody purity was available for any of the antibodies used in this thesis.

Table 2.2 Effect of anti-BNP antibody on the signal intensity ratio (/ACTH) of synthetic BNP molecular forms (/ACTH) as spiked into LC-MS grade water (positive control) and also in iPSC-derived cardiomyocytes

		Positive Control			iPSC-derived cardiomyocyte		
		BNP 5-32	BNP 4-32	BNP 3-32	BNP 5-32	BNP 4-32	BNP 3-32
Signal Intensity (/ACTH)	Ky-hBNP II	2.60	2.15	1.35	5.75	3.55	5.15
	Sekisui 0	0.45	0.15	0.35	0.20	0.15	0.25
	Sekisui 2	1.35	1.25	0.70	3.85	2.35	5.00
	Sekisui 7	3.80	3.30	1.90	7.30	3.90	8.90

Sekisui 7 performed as the best anti-BNP antibody showing higher ratios across all of the molecular forms in the positive control and in the iPSC-derived cardiomyocytes. This antibody also displayed minimal background interference and lower levels of non-specific binding. Sekisui 7 was the chosen antibody for further experiments alongside the second best performing antibody, Ky-hBNP II.

2.2.3 ACTH concentration

Initially 1 fmol/µL ACTH was used as the signal peptide in previous experiments, however it was noticed that due to the very strong signal intensities of some BNP molecular forms when anti-BNP antibodies were used ACTH was shown on the spectra as a small peak, at times difficult to notice. It was thought that a higher concentration of ACTH would balance the strength of BNP molecular form peak and ACTH peak and allow easier detection of the peaks. ACTH at 5fmol/µL and 10fmol/µL were therefore used to see their effects on the signal intensity of ACTH and subsequent BNP molecular form peaks in iPSC-derived cardiomyocytes. Sekisui 7 and Ky-hBNP II were the anti-BNP antibodies used in this experiment. The results are shown in Table 2.3.

Table 2.3 Effect of ACTH concentration on the signal intensity ratio (/ACTH) of synthetic BNP molecular forms (/ACTH) as spiked into LC-MS grade water (positive control) and also in iPSC-derived cardiomyocytes

			Positive Control			iPSC-derived cardiomyocyte		
			BNP 5-32	BNP 4-32	BNP 3-32	BNP 5-32	BNP 4-32	BNP 3-32
Signal Intensity (/ACTH)	5fmol/µL	Ky-hBNP II	0.17	0.10	0.06	0.15	0.11	0.13
		Sekisui 7	1.17	0.70	0.39	1.53	0.59	1.33
	10fmol/µL	Ky-hBNP II	0.09	0.06	0.01	0.07	0.03	0.07
		Sekisui 7	0.37	0.22	0.14	0.78	0.26	0.63

At 5 fmol/µL the detection of BNP molecular forms using both antibodies showed a much greater ratio. At 5 fmol/µL ACTH, it allowed easy detection of BNP molecular forms compared to 10 fmol/µL, and compared to ACTH concentration at 1 fmol/µL it showed lower levels of background interference due to the strength of ACTH as well as easily detectable ACTH. ACTH concentration of 5 fmol/µL was used in further studies.

2.2.4 Detection of BNP in healthy human plasma

In healthy individuals, BNP levels are much lower than the cut off value. Plasma samples from healthy individuals were obtained (n=3) to investigate whether BNP molecular forms are detectable. Healthy individuals were defined as free-from disease. 100µL of healthy plasma was added to 900µL Buffer A, and Sekisui 7 and Ky-hBNP II were used as anti-BNP antibodies. The results are shown in Table 2.4.

Table 2.4 Detection of BNP molecular forms in healthy human plasma.

		Healthy Plasma		
		BNP 5-32	BNP 4-32	BNP 3-32
Signal Intensity (/ACTH)	Ky-hBNP II	0.00	0.00	0.00
	Sekisui 7	0.00	0.00	0.00

Results showed that due to the very low levels of BNP in healthy individuals, no molecular forms were detected by both antibodies.

2.2.5 Limit of detection (LOD)

After successfully detecting BNP at low concentration in synthetic BNP dissolved in water and failing to detect BNP in healthy plasma, spiking water and healthy plasma with synthetic BNP molecular forms was used to investigate whether BNP could actually be detected in plasma or whether there are other factors to consider when detecting in plasma. The limit of detection (LOD) was determined by analysing a range of BNP molecular form concentrations. A signal:noise >3 (S:N>3) was used to define the LOD (Figures 2.2 and 2.3).

100µL of healthy plasma was added to 900µL Buffer A which was spiked with 1µL synthetic BNP molecular forms (3-32, 4-32 and 5-32 at 100 fmol/mL). Sekisui 7 was used as the antibody of choice.

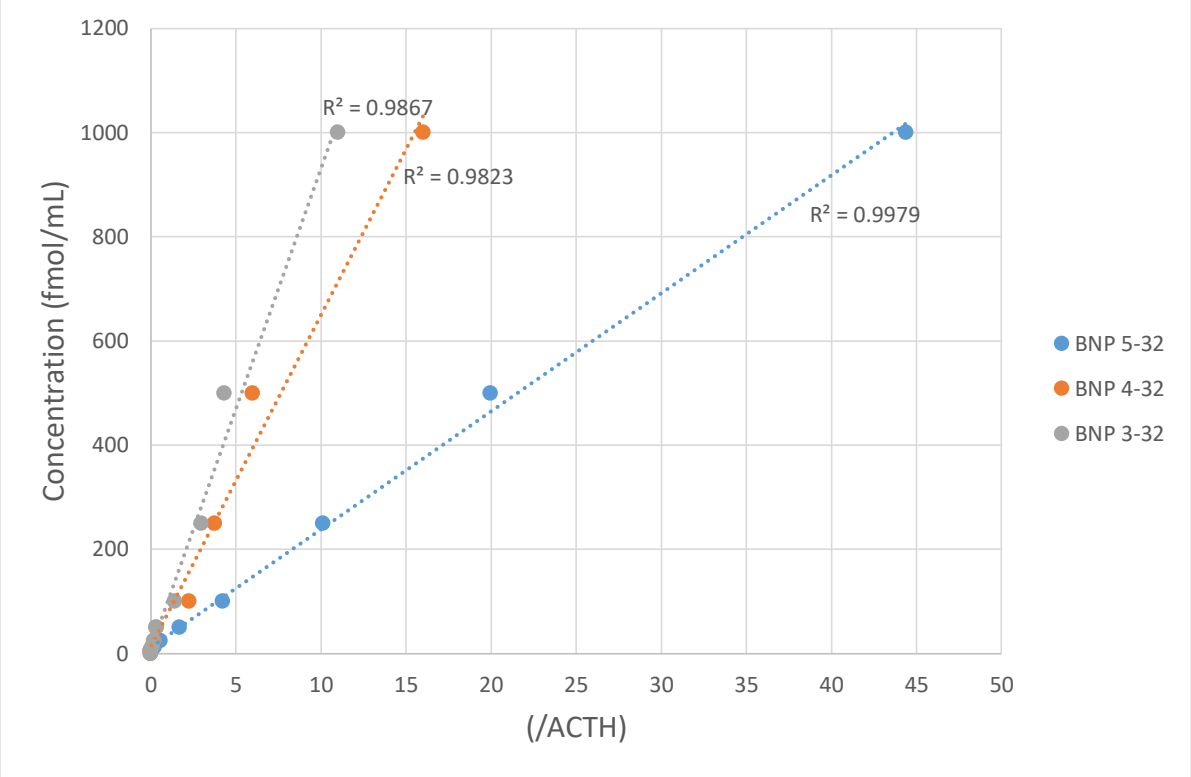


Figure 2.2 Concentration curve for BNP molecular forms (BNP 5-32, 4-32, 3-32) spiked into water at different concentrations (1000-0 fmol/mL) to determine the LOD

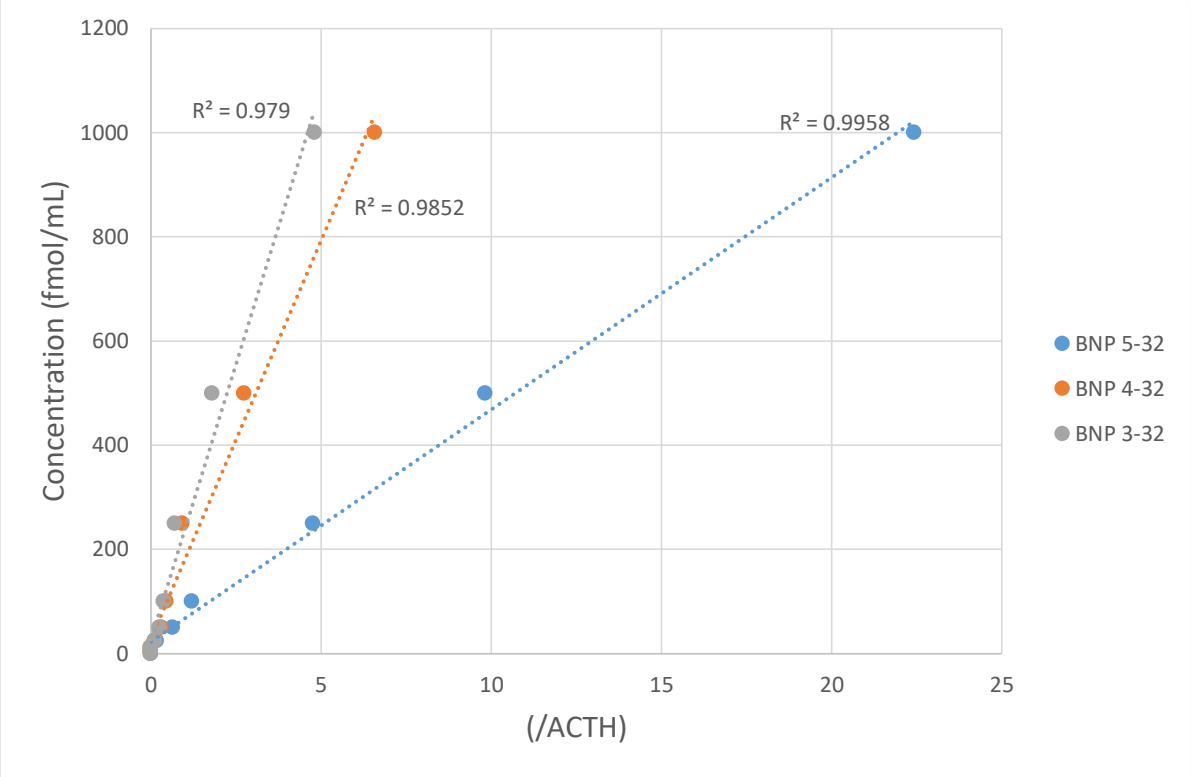


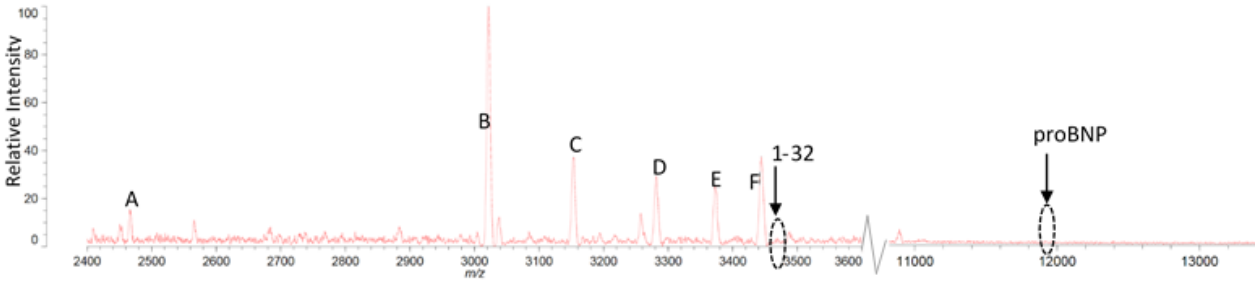
Figure 2.3 Concentration curve for BNP molecular forms (BNP 5-32, 4-32, 3-32) spiked into healthy plasma at different concentrations (1000-0 fmol/mL) to determine the LOD

The results showed that in water the lowest detectable concentration of the BNP molecular forms was at 12.5 fmol/mL (37.5 pg/mL) whereas in plasma this was at 25 fmol/mL (75.6 pg/mL). In addition to this, the detection in water was approximately two-fold greater than in plasma highlighting the difficulties in sample preparation in medium such as plasma.

These parameters finalised the methods used for further studies. The incubation times were set at 1 hour, Sekisui 7 anti-BNP antibody was used and ACTH concentration at 5fmol/µL. These methods thus showed that BNP molecular forms were detectable at levels below the BNP cut-off value of 100pg/mL.

2.2.6 Mass spectrometry analysis and immunoreactivity with other molecular forms

The antibodies used in this study allowed selective immunocapture of BNP molecular forms 5-32, 4-32 and 3-32. The antibody used in this study recognises and binds to the ring region of BNP, therefore any molecular form of BNP, including BNP and proBNP itself are recognisable by the antibody. To investigate which molecular forms are detectable using this antibody, plasma samples from the acute HF and acute MI studies were studied and interrogated for the possibility of minute levels of other molecular forms. Results showed that BNP 1-32, proBNP and other alternative BNP molecular forms were not detected for these samples using our experimental protocol. An example mass spectrum from a patient sample showing the presence of BNP molecular forms and absence of BNP 1-32 and proBNP can be found in Figure 2.4.



	Expected mass (monoisotopic mass)	Actual mass $[M+H]^+$
A – ACTH	2464.20	2466.84
B – BNP 5-32	3022.51	3021.92
C – BNP 4-32	3153.71	3153.31
D – BNP 3-32	3281.88	3281.24
E	Unknown peak, detected in previously reported study (Fujimoto et al., 2013)	3373.06
F	Unknown peak, detected in previously reported study (Fujimoto et al., 2013)	3444.52
1-32	3466.08	Undetected
proBNP	11905.55	Undetected

Figure 2.4 Example spectra confirmation of BNP molecular forms and ACTH detection in acute MI plasma, and non-detection of BNP 1-32 and proBNP

ACTH, Adrenocorticotrophic hormone; BNP, B-type natriuretic peptide, *m/z*, mass-to-charge ratio

Note

For method development, no preparative replicates were run and thus no error bars are shown in the data. All measurements as reported as averages of duplicates as samples were spotted in duplicate onto the MALDI target plate.

The ACTH spectrum consistently demonstrated a gain in mass or around 2 daltons and BNP 5-32 showed a consistent loss in mass of around 2 daltons. As seen in the figure, both BNP 4-32 and BNP 3-32 were consistently more accurate and close to the expected mass. A possible suggestion for this could be due to effects of suppression between ACTH and BNP 5-32 that could have caused the shift in mass, but not affected BNP 4-32 and BNP 3-32.

2.3 Method

2.3.1 Materials and reagents

Synthetic BNP molecular forms (BNP 3-32, 4-32 and 5-32) were produced by Peptide Institute Inc. (Osaka, Japan), and the anti-BNP monoclonal antibodies KY-hBNP II and Sekisui 33240, 33242, 33247 were kindly donated by Shionogi & Co (Osaka, Japan) and Sekisui Medical Co. (Tokyo, Japan), respectively.

For the immunocapture sample preparation, Buffer A-NP40* (0.1% NP40 Igepal CA630 in phosphate buffer saline (PBS)), PBS*, NH_4HCO_3*, and bovine serum albumin (BSA) were all purchased from (Sigma-Aldrich Company Ltd., Dorset, UK). Dynabeads® M-280 Sheep-Anti Mouse IgG and TFA were both purchased from Fisher Scientific (Loughborough, UK).

For MALDI-MS, ACTH was purchased from Sigma-Aldrich Company Ltd. (Dorset, UK) and 10 mg/mL Matrix (α-CHCA and DHB) from LaserBio Labs (Sophia Antipolis, France).

*Dilution of buffers (Buffer A-NP40, PBS and NH_4HCO_3) were made using Optima™ LC-MS grade water purchased from Fisher Scientific (Loughborough, UK).

2.3.2 Sample preparation

Positive Control

Each BNP peptide molecular form (1pmol/mL- synthetic 3-32, 4-32 and 5-32) was diluted in deionised water at 100fmol/mL. 1µL was added to Buffer A in a final volume of 1mL.

Negative Control

100µL BSA was added to 900µL Buffer A.

Sample

100µL of sample was added to 900µL Buffer A.

2.3.3 Immunoprecipitation

10mg/mL Dynabeads® M-280 Sheep-Anti Mouse IgG (5µL/sample) were washed four times with 1mL PBS using a magnetic particle concentrator (DynaMag™-2 Life Technologies) and the supernatant removed, before resuspending in 1mL PBS and adding anti-BNP antibody (350ng/sample). The samples were then incubated at room temperature on a rotator (4rpm) for 60 minutes. Unbound antibody was removed by washing five times with 1mL PBS using a magnetic particle concentrator.

20µL of Antibody was added to each sample and incubated at room temperature on a rotator (4rpm) for 60 minutes; allowing the formation of antigen-antibody-magnetic beads complex. Unbound antigen was then washed four times with 1mL PBS using a magnetic particle concentrator, removing the supernatant, before washing the samples using the same process with 1mL 20mM NH_4HCO_3 twice and then once with 1mL 2mM NH_4HCO_3. The samples were centrifuged for 5 minutes at 14000rpm removing the residual supernatant and eluted using a magnetic particle concentrator with 3µL of 0.1% TFA.

2.3.4 MALDI-ToF MS

Mass spectrometry analysis was performed on the samples by applying 0.75 µL of each sample, ACTH (signal peptide, calibration standard) and 10 mg/mL matrix (DHB and α-CHCA made in a 1:1 ratio) onto the MALDI target plate. The plate was incubated at 37°C before mass spectrometry measurement using the AXIMA Confidence (Shimadzu Corporation) and data analysis. The Axima Confidence was operating in linear mode and spectra obtained showing on average between 150 and 200 profiles. Each sample was spotted in duplicate and two spectra were obtained from each spot, giving a total of four spectra per sample from which averages were obtained. Semi-quantitative analysis was performed by expressing the centroid peaks data (signal intensity) of the BNP molecular forms as a ratio against the signal intensity of ACTH as shown in Figure 2.5. A +16 adduct was detected alongside the molecular forms, but not ACTH (shown as red circles in Figure 2.5 (A)). These peaks were present in all BNP molecular forms across all of the studies presented in this thesis. Despite this, these adducts were not used in the quantitation of BNP molecular forms because these adducts showed no correlations

with the molecular forms when the area under the peak or % signal intensity was considered, as shown in Figure 2.5 (A).

Average mass-to-charge (m/z ±3) for ACTH= 2464.20, BNP 5-32= 3022.51, BNP 4-32= 3153.71, BNP 3-32= 3281.88.

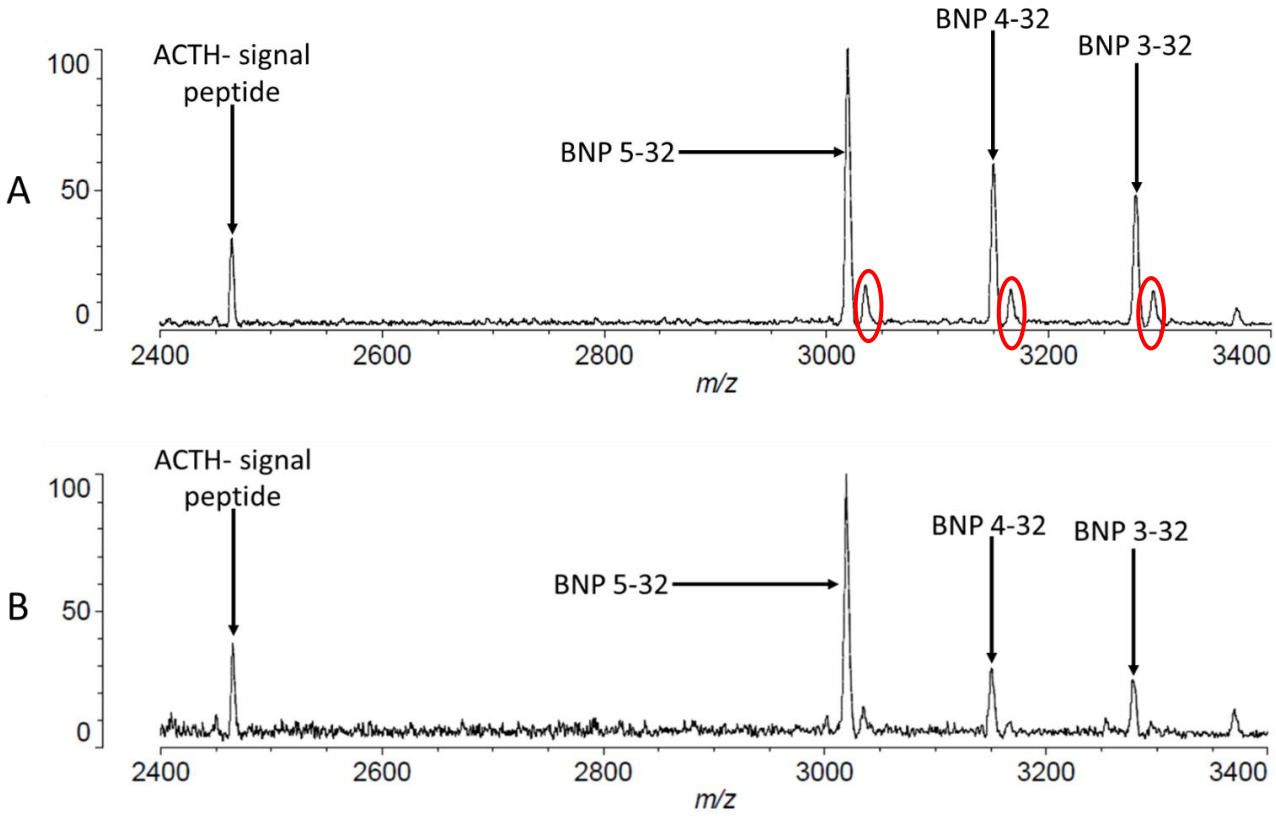


Figure 2.5 Example mass spectra of (A) Positive control using synthetic BNP 5-32, BNP 4-32 and BNP 3-32 and (B) acute HF patient plasma showing detectable peaks relating to ACTH signal peptide, BNP 5-32, BNP 4-32 and BNP 3-32

Red circles show the +16 adduct on the molecular forms in (A). The adducts are also present in (B)

ACTH, Adrenocorticotrophic hormone; BNP, B-type natriuretic peptide

ACTH (18-39) was used as the internal standard due to its ease of detection and its *m/z* being within the same spectrum as the BNP molecular forms (*m/z* 2466.84 and 3021.92-3281.24 respectively), without suppressing the signal intensities of the molecular forms. Alongside ACTH (18-39), other internal standards are commonly used with MALDI applications including oxidised insulin B chain (*m/z* 3495.89), bradykinin fragment 1-7 (*m/z* 756.40) and angiotensin II (*m/z* 1045.54).

For quantitation on the mass spectrometer, the quality of data obtained depends on several factors including;

- Sample preparation and positioning on the target plate
- Calibration of the instrument for accurate mass measurement
- Data processing to reduce baseline noise and improve the signal-to-noise ratio

Calibration points used for the mass spectrometer in this thesis were as per discussion with Shimadzu's technical support team. Calibration was based upon small mass peptides (m/z range 0-3500). Calibration points used (m/z expected) were angiotensin II (m/z 1046.54), ACTH 18-39 (m/z 2465.20) and ACTH 7-38 (m/z 3657.93). An example calibrated mass spectrum is shown in Figure 2.6.

Further peaks were shown for angiotensin I (m/z 1296.69), Glu-1-fibrinogen (m/z 1570.68), N-acetyl renin (m/z 1800.94) and ACTH 1-17 (m/z 2093.09).

Data processing and acquisition of spectra is shown in Figure 2.7. The laser was fired at 80 (power), as recommended by the manufacturer. Peaks were processed with baseline subtraction to remove non-BNP molecular form peak that could otherwise supress the signal of the peak of interest, and smoothed. Despite baseline subtraction to remove/reduce very low frequency noise from the spectrum, background interference was still present in some samples such as that sown in Figure 5.2. This background was not further subtracted, and its occurrence could be due to the operation of the laser at a too high power for a particular sample or issues with the sample preparation (immunoprecipitation). Even though a thorough sample preparation procedure was carried out, due to the nature of plasma and salts used in the clean-up, background can

still remain (Figure 2.7 (A)). Peak detection was reported as area between the curve crossing the threshold (threshold centroid) (Figure 2.7 (B)).

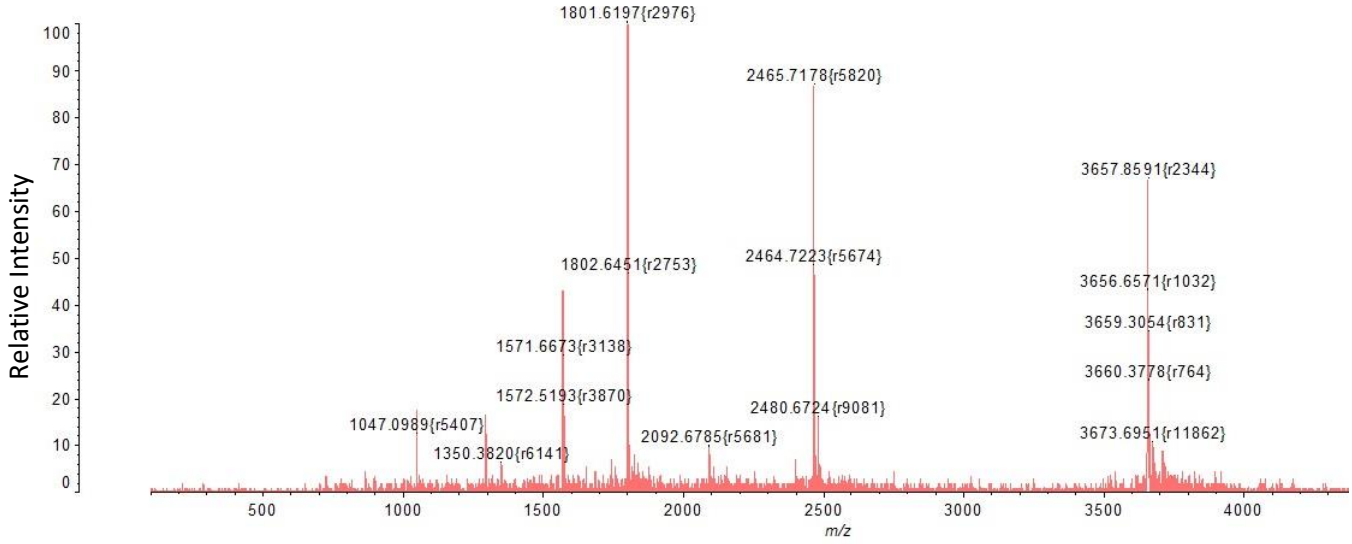
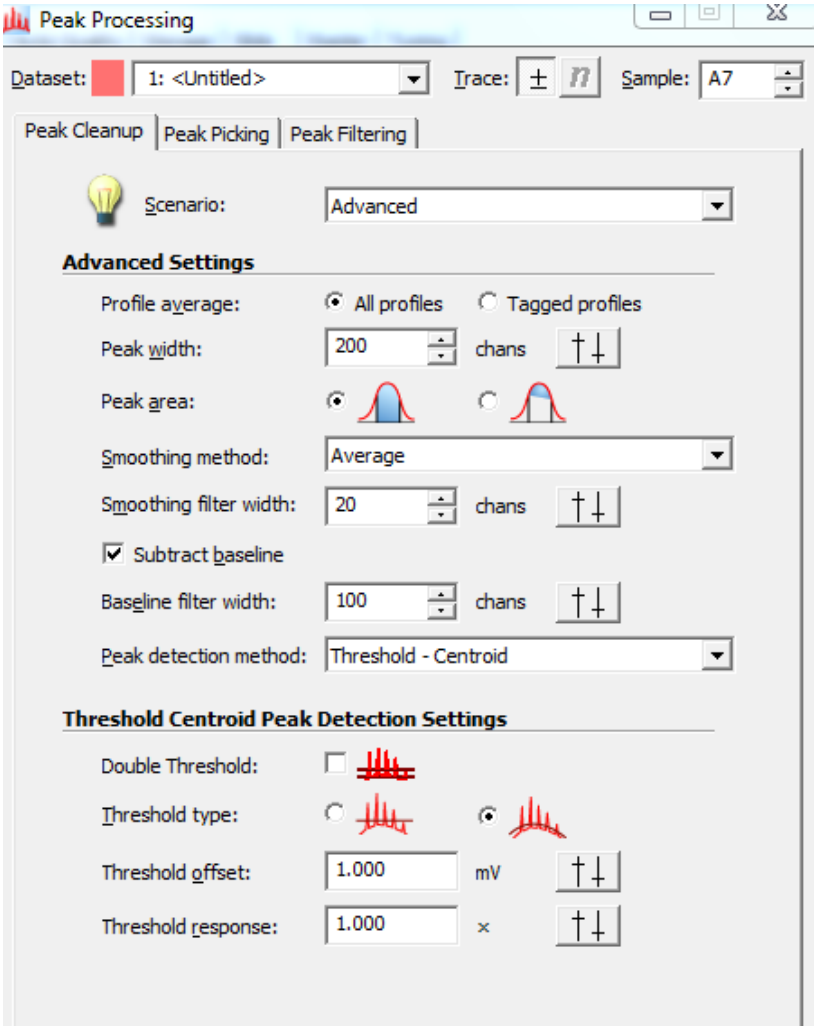


Figure 2.6 Example mass spectrum showing the calibration points used

A



B

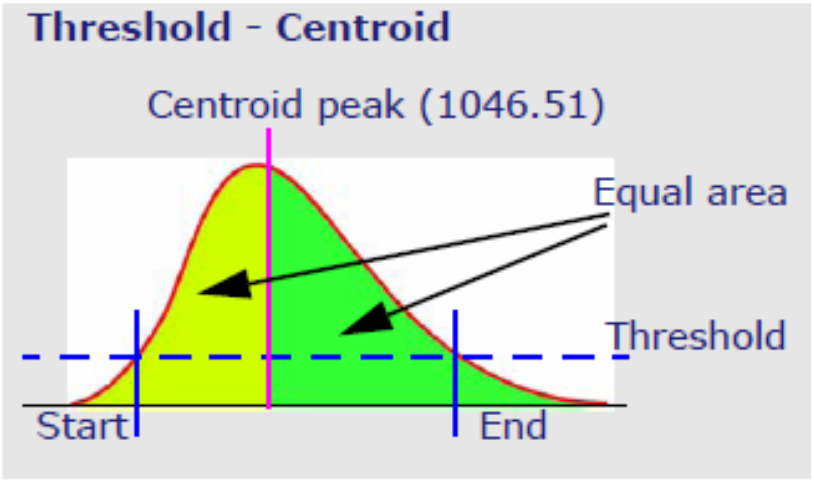


Figure 2.7 Showing the parameters used on the Shimadzu Axima Confidence for data processing and acquisition for (A) peak processing and (B) peak detection

2.3.5 Ethics

Three clinical studies were conducted for this thesis, and the method development which involved the use of human plasma. All the studies were conducted as per the declaration of Helsinki and local rules of research and governance were followed. Some examples of the ethical approvals and consent forms are provided below.

The BIOSTAT-CHF study had individual ethics certified at each centre (69 centres in total). The study protocol was ethically approved on the 17th June 2010, MREC Number 10/S1402/39.

CONSENT FORM

Title of Project: Studies of blood cells and haemostatic markers

Name of Researcher: Professor [redacted]

Please initial box

1.	I confirm that I have read and understand the information sheet for the above study (dated 03.08.2016) and have had the opportunity to ask questions.	ZI
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason.	ZI
3.	I agree to take part in the above study.	ZI

Name of subject	Date	Signature
M Zubair Israr	4/10/16	

Name of Person taking consent (if different from researcher)	Date	Signature
[redacted]	4.10.16	

Researcher	Date	Signature
[redacted]	7/10/16	

Derbyshire Research Ethics Committee
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07 September 2006
Reissued 3rd October 2006 to show updated documents

Prof Leong L Ng
Professor & Honorary Consultant in Medicine & Therapeutics
University of Leicester
Department of Cardiovascular Sciences, Pharmacology & Therapeutics Div
Level 4, Robert Kilpatrick Clinical Sciences Building
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LE2 7LX

Dear Prof Ng

Full title of study: **Biochemical Markers involved in the prediction of future cardiac events in Acute Heart Failure**

REC reference number: **06/Q2401/112**

Thank you for your letter of 31 August 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Continued/

Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		07 August 2006
Investigator CV	Key Investigator (Dr T Ali)	
Investigator CV	Key Investigator (Dr Chin)	
Investigator CV	Key Investigator (Dr Squire)	
Investigator CV	CI (Professor Ng)	
Protocol	6	21 August 2006
Covering Letter		29 June 2006
Peer Review		13 April 2006
GP/Consultant Information Sheets	5 (letter)	07 August 2006
Participant Information Sheet	7	31 August 2006
Participant Consent Form	7	31 August 2006
Response to Request for Further Information	3rd Response	31 August 2006
Response to Request for Further Information	1st Response	07 August 2006
Data monitoring form [DATA COLLECTION FORM]		
Response to Request for Further Information	2nd Response	21st August 2006

Research governance approval
The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q2401/112	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Peter Korczak
Chairman

Email: jenny.hancock@derwentsharedservices.nhs.uk

Enclosures: *Standard approval conditions*
Site approval form

Copy to: Professor David Rowbotham
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24 August 2010

Professor Leong L. Ng
Professor of Medicine and Therapeutics
University of Leicester
School of Medicine, Department of Cardiovascular Sciences
Pharmacology and Therapeutics Group
Level 4, Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester, LE2 7LX

Dear Professor L. Ng,

Study title: **Risk Stratification of myocardial infarction using cardiac peptides**
REC reference: **7119**
Amendment number: **5**
Amendment date: **23 July 2010**

The above amendment was reviewed at the meeting of the Sub-Committee held on 19 August 2010.

Ethical opinion

Favourable Opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
GCP certificate for additional investigator		28 June 2010
Participant Consent Form	2	23 July 2010
Participant Information Sheet	2	23 July 2010
Investigator CV		
Notice of Substantial Amendment (non-CTIMPs) - changes to PIS / CF and additional investigator	5	23 July 2010
Covering Letter		23 July 2010

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

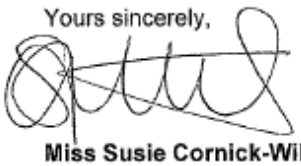
All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

7119: Please quote this number on all correspondence

Yours sincerely,



Miss Susie Cornick-Willis
Committee Co-ordinator

E-mail: susie.cornick-willis@nottspct.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mr Graham Hewitt - University of Leicester*

R&D office for NHS care organisation at lead site - UHL

Leicestershire, Northamptonshire & Rutland Research Ethics Committee 2

Attendance at Sub-Committee of the REC meeting on 19 August 2010

Name	*Profession*	*Capacity*
Mr Geoff Dickens	Research Manager	Expert
Mr Ken Willis	Medical Devices Manager	Lay

Also in attendance:

Name	*Position (or reason for attending)*
Miss Jessica Chatrie	Administrative Officer
Miss Susie Cornick-Willis	Committee Co-ordinator

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH AND DEVELOPMENT
Director: Professor D Rowbotham
Business Manager: Dr N J Seare
Service Manager: Mr M Roberts

Co-ordinator: N Turner
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Leicester General Hospital
Gwendolen Road
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Fax: 0116 2584666
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30 October 2003

Dr Leong L. Ng
Professor of Medicine & Therapeutics & Honorary Consultant Physician
Department of Medicine & Therapeutics
Robert Kilpatrick Clinical Sciences Building
LRI

Dear Dr Ng

RE: **UHL Ref. 9123** *[Please quote this number in all correspondence]*
Risk stratification of myocardial infarction using cardiac peptides.

We have now been notified by the Ethics Committee that this project has been given a favourable opinion by the Ethics Committee (please see the attached letter from the Ethics Committee).

Since all other aspects of your UHL R+D notification are complete, I now have pleasure in confirming full approval of the project on behalf of University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary.

This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your notification form.

The project is also now covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

In the meantime, in order to keep our records up to date, could you please notify the Research Office if there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.

Yours sincerely

Mr Michael Roberts
Service Manager for Research and Development

CHAPTER THREE

ACUTE HEART FAILURE STUDY

This chapter has been published elsewhere

Suzuki T*, **Israr MZ***, Heaney LM, Takaoka M, Squire IB, Ng LL. 2017. Prognostic role of molecular forms of B-type natriuretic peptide in acute heart failure. Clinical Chemistry, 63:880-886.

3. ACUTE HEART FAILURE STUDY

3.1 Introduction

3.1.1 Introduction and Rationale

Both BNP (Stolfo et al., 2017, Lourenço et al., 2017) and NT-proBNP (Januzzi et al., 2006, Kang et al., 2015, Huang et al., 2016) have been well documented to show utility in short- and long-term prognosis in acute HF. However, an important limitation of these markers is that circulating levels may become elevated in response to alternative disorders such as renal dysfunction, left ventricular hypertrophy and right ventricular dysfunction (Luchner et al., 2000).

Although widely used in clinical analysis, BNP has been shown to have a relatively short-half life (approximately 20 minutes) when present in circulation (Potter, 2011) and further studies have shown that concentrations of BNP 1-32 are very low in patients with symptomatic HF and degradation of BNP results in molecular forms and common detectable molecular forms include 5-32, 4-32 and 3-32 as well as many other forms (Niederkofler et al., 2008).

In addition to this, Miller et al. (2011) showed that the combination of these molecular forms in circulation showed a better representation of clinical BNP measurements, than BNP measured in isolation. Clinically measured BNP was also shown to correlate better with MS degradation molecular forms and relatively poorly with the MS signal of the intact BNP molecular ion (BNP-32), suggesting that clinically measured BNP is composed of a collation of such molecular forms (Miller et al., 2011). A more recent paper then showed that BNP molecular forms have been implicated in ischaemic heart disease, with BNP 5-32 being suggestive as a role in the pathogenesis of restenosis (Fujimoto et al., 2013).

Currently there is no data available investigating the prognostic role of BNP molecular forms and outcomes in hospitalised patients with acute HF.

3.1.2 Aims and Hypothesis

- Investigate the associations of BNP molecular forms in patients admitted with acute HF
- Assess the applications of these measurements for use in prognostic risk prediction

Using the developed MALDI-ToF-MS immunoassay method BNP molecular forms are expected to be detected in patient plasma and show similar prognostic abilities as the clinically measured biomarker, NT-proBNP.

3.2 Methods

3.2.1 Study population

This cohort has been previously used and therefore the sample collection and study population criterion had been arranged prior.

Patients with acute HF were admitted to the University Hospitals of Leicester between February 2006 and August 2011 and enrolled. Each patient consented to have blood samples taken and outcomes surveyed. This study was approved by the local ethics committee and adhered to the Declaration of Helsinki. Diagnosis of acute HF was made on the clinical signs and symptoms including pulmonary oedema, peripheral oedema or elevated jugular venous pressure and progressively worsening or new onset of shortness of breath (McMurray et al., 2012). Patients excluded from the study included those with previous history of cancer or renal replacement therapy; any surgical procedure within the previous month; presence of cardiogenic shock, sepsis, pneumonia; acute coronary syndromes; and inability to consent (eg, dementia). Patients were not excluded on values of preserved/reduced ejection fraction where echocardiograms were performed.

For prognostic investigations, the primary endpoints were all-cause mortality (death) and a composite of death and rehospitalisation due to heart failure (death/HF) at 6 months and 1 year. All surviving patients were followed up for a minimum of 1 year, with all outcome data obtained from hospital records. In cases where multiple events occurred, the time to the first event was counted as the outcome. Outcome data surpassing 1 year of follow up are not included in this report. Inclusion and endpoint evaluations were determined by an independent cardiologist. Glomerular filtration rate (eGFR) was estimated using the simplified modified diet in renal disease formula (Suzuki et al., 2016).

This cohort was initially designed for the investigation of biochemical markers in the prediction of future cardiac events in acute HF. The study presented in this thesis lies within the remit of the initial intentions of the study, looking at prognosis in acute HF. First of all, because BNP/NT-proBNP is a gold standard biomarker for HF, this study was

initially selected due to the specificity of BNP/NT-proBNP as a HF biomarker. This allows direct comparisons between BNP and BNP molecular forms to be made. Secondly, the patients in this study had acute HF, therefore the concentrations of NT-proBNP are excessively elevated allowing for an expected high detection of BNP molecular forms. The latter strength of this cohort was particularly useful as a 'proof of concept' study as this study was the first to show the prognostic role of BNP molecular forms in HF. Lastly, this study had a large sample number (n=904) allowing to better determine the impact of the study (i.e confidence intervals and margins of error).

The first obvious limitation of this study was the age of the plasma samples. Although the samples were collected, stored in aliquots and well preserved, these samples were 4-10 years old. It could well be the case that some of the peptide present may have degraded over time, even though an appropriate protease inhibitor was used. It is worth noting that commonly, plasma or serum is stored at -80°C. It is also important not to allow samples to undergo too many freeze-thaw processes such that particularly after two cycles results showed increasing changes in results. Considering this, long-term storage for 4-years had minimal effect on the results (Vaught, 2006, Mitchell et al., 2005). Also, for direct comparisons for BNP molecular forms, BNP concentrations were necessary. However, for this study BNP concentrations were unavailable and NT-proBNP was measured instead. As the applicability of BNP molecular forms showed similar prognostic ability to NT-proBNP, additional measurement of BNP levels was not considered further beneficial. Logistical implications of further analyses were also weighed (e.g. cost, sample provision, etc.). Even considering this, BNP vs BNP molecular forms comparisons would have been more advantageous. Although samples were collected within 24 hours post-admission, the time (hours) of sample collection is unknown.

3.2.2 Sample collection

Samples were collected within 24 hours post-admission to hospital with acute HF. Blood was collected in pre-chilled tubes containing EDTA and aprotonin, centrifuged at 1500g for 20 min at 4°C. Plasma was aliquoted and stored at -80°C until analysis. For analysis, samples were thawed at 37°C prepared, and analysed immediately.

3.2.3 BNP molecular form measurements

BNP molecular forms were measured and the results were compared to NT-proBNP values previously measured in all patients for other studies using a sandwich immunoassay (Omland et al., 2002). Inter-assay relative standard deviations for daily control experiments across the course of the study (60 days) for synthetic BNP 5-32, BNP 4-32 and BNP 3-32 were 6.3%, 8.0% and 10.3 %, respectively. Distribution and variation of daily control experiments can be visualised in Figure 3.1. Relative standard deviation (RSD) was controlled within an average of 10% for the molecular forms, as shown in Figure 3.1 the average RSD = 8.2%.

3.2.4 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (V.22, IBM Corp, Armonk, New York, USA). Correlations between BNP molecular forms and with other clinical variables were performed using Spearman's correlations. Multivariate and univariate models were made to evaluate independent predictors of death and death/HF using Cox proportional hazard regressions analyses. Molecular BNP forms and NT-proBNP values were log transformed and normalised to 1 standard deviation so the hazard ratios refer to the Z-transformed values.

Binary logistic regression was used to test the predictive value of log molecular BNP forms and log NT-proBNP values for in-hospital mortality and further adjusted for the ADHERE, OPTIMIZE-HF and GWTG-HF clinical risk scores (Adams et al., 2005, Fonarow et al., 2008a, Peterson et al., 2010). The ADHERE score was assessed as both a categorical value against the first reference grouping and as a continuous variable from the formula for log odds mortality (Adams et al., 2005). ADHERE groupings were allocated according to the ADHERE registry model ranging from 1 (lowest risk) to 5 (highest risk) (Adams et al., 2005). Kaplan-Meier survival curves were generated to evaluate between the molecular forms and outcomes. Mantel-Cox log rank tests were used to compare event-free survival after stratification of biomarkers by tertiles. A p-value of <0.05 was considered to be statistically significant.

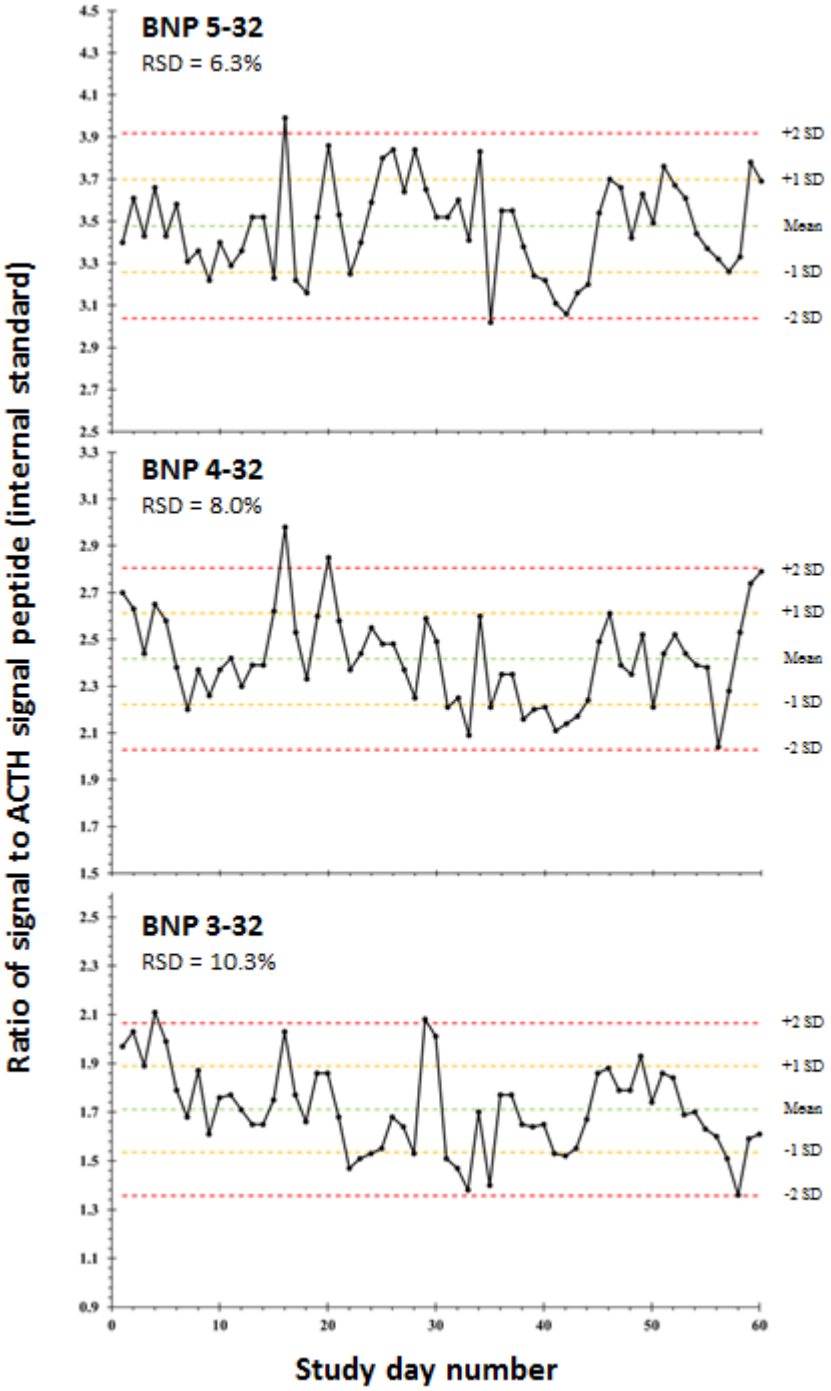


Figure 3.1 Relative Standard Deviation for synthetic BNP 5-32, BNP 4-32, and BNP 3-32 used as the positive control throughout the course of the study. Average RSD = 8.2% was less than the 10% limit and within three SD.

ACTH, Adrenocorticotrophic hormone; BNP, B-type natriuretic peptide; RSD, Relative standard deviation

3.3 Results

3.3.1 Cohort characteristics and demographics

Plasma from 904 patients were analysed for the presence of BNP molecular forms. Mass spectral peaks for BNP molecular forms were detectable in a total of 838 samples (93%), with the remaining 66 patient samples excluded from further analyses. A total of 332 events were recorded during the 1 year follow up period, with 60 deaths during in-hospital care, 160 deaths post-discharge and 105 readmissions to hospital due to HF.

Analysis of trends in this cohort showed that the majority of the cohort were male and a median age of 78 years. The median NT-proBNP was extensively elevated at 2285 pmol/L (19194 pg/mL, cut off = 400 pg/mL), indicating acute HF. A breakdown of the measured endpoints, along with the clinical demographics for the patient cohort can be found in Table 3.1.

3.3.2 BNP molecular forms and associated clinical measurements

Univariate analysis showed clinical variables that correlated to one or more of the BNP molecular forms to be blood urea, eGFR, age, systolic blood pressure, respiratory rate and sodium, as detailed in Table 3.2. All BNP molecular forms were observed to be correlated to NT-proBNP, and strongly correlated between themselves as shown in Figure 3.2. The strongest level of correlation was observed between BNP 5-32 and BNP 4-32 (r_s = 0.863), however all three molecular forms showed strong levels of correlations between themselves (r_s = 0.809-0.863). All three BNP molecular forms showed a modestly positive correlation with NT-proBNP (r_s = 0.438-0.557), with BNP 5-32 showing the best correlation (r_s = 0.557). Many of the samples were dispersed away from the line of best fit and a lot of the samples showed that high levels of NT-proBNP did not necessarily show high levels of molecular forms of BNP. Correlations with markers of renal dysfunction (urea and eGFR) showed that BNP 5-32, 4-32 and 3-32 possessed a moderately reduced association than when compared against the relationship seen for NT-proBNP; for urea r_s = 0.187-0.246 for the BNP molecular forms and 0.369 for NT-proBNP, and for eGFR r_s = -0.158- -0.211 for the BNP molecular forms and -0.284 for NT-proBNP.

A linear regression model was performed to investigate the independent predictors of BNP molecular forms (log-transformed) and NT-proBNP. Independent predictors for each of the BNP molecular forms can be found in Table 3.3. The results showed that eGFR was independently predictive of all BNP molecular forms ($p \leq 0.011$), and BNP 5-32 and NT-proBNP were independently predicted by blood urea levels ($p \leq 0.019$) thus illustrating that renal dysfunction is indicated in the presence of both BNP molecular forms and NT-proBNP. Interestingly, only BNP 5-32 and NT-proBNP were influenced by age ($p \leq 0.002$) and BNP 4-32 and 3-32 the only biomarkers influenced by current severity of heart failure (as measured by NYHA class, $p \leq 0.006$).

Table 3.1 Patient demographics for acute HF patients at the time of admission to hospital

Age (yrs)	78 (70-84)
Male	61%
Systolic BP (mmHg)	133 (115-150)
Diastolic BP (mmHg)	75 (65-85)
Heart rate (beats/min)	88 (73-105)
de novo HF	34%
Past history IHD	27%
Past history diabetes	34%
Past history HTN	58%
Past history COPD	10%
Past history hyperlipidemia	25%
Current smoker	9%
Orthopnea	54%
Oedema	64%
Raised JVP	56%
Pulmonary oedema	32%
NYHA Class IV	55%
Atrial fibrillation	47%
Respiratory rate (breaths/min)	22 (18-25)
Urea (mmol/L)	8.9 (6.6-12.8)
Creatinine (µmol/L)	112 (91-141)
eGFR (mL/min/1.73m^2)	52 (39-68)
Na^+ (mmol/L)	138 (135-141)
K^+ (mmol/L)	4.4 (4.0-4.7)
Haemoglobin (g/L)	123 (107-137)
NT-proBNP (pmol/L)	2285 (1140-4036)
ADHERE group 3-5	16.7%
OPTIMIZE-HF score	35 (30-40)
GWTG-HF score	43 (38-48)
BNP 5-32	0.9 (0.4-1.6)
BNP 4-32	0.5 (0.2-0.7)
BNP 3-32	0.5 (0.3-0.8)
Endpoints	
In-hospital death	60
Death at 1 year	227
Death/HF at 1 year	332
Death at 6 months	168
Death/HF at 6 months	267

Combined data is shown as median (interquartile range) for continuous variables and as a % for categorical. BNP molecular forms are reported as MS ion signal intensity ratio against an internal reference standard (/ACTH) (5 fmol/µL)

BNP, B-type natriuretic peptide; BP, blood pressure; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; HF, heart failure; HTN, hypertension; IHD, ischaemic heart disease; JVP, jugular venous pressure; NT-proBNP, N-terminal pro B-type natriuretic peptide; NYHA, New York Heart Association

(Peterson et al., 2010, Fonarow et al., 2008a, Abraham et al., 2008)

Table 3.2 BNP molecular forms and associated clinical factors

	BNP 5-32		BNP 4-32		BNP 3-32		NT-proBNP	
	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value
Urea	0.246	<0.001	0.213	<0.001	0.187	<0.001	0.369	<0.001
eGFR	-0.211	<0.001	-0.183	<0.001	-0.158	<0.001	-0.284	<0.001
Age	0.132	<0.001	0.106	0.002	0.047	0.171	0.133	<0.001
Systolic BP	-0.100	0.005	-0.089	0.013	-0.080	0.026	-0.164	<0.001
Respiratory Rate	0.075	0.046	0.056	0.138	0.043	0.251	-0.045	0.232
Sodium	-0.076	0.034	-0.053	0.139	-0.016	0.664	-0.047	0.190
BNP 5-32			0.863	<0.001	0.809	<0.001	0.557	<0.001
BNP 4-32					0.813	<0.001	0.463	<0.001
BNP 3-32							0.438	<0.001

Table showing the associations and correlations between traditional clinical factors and BNP molecular forms. BNP molecular forms showed a strong positive correlation between themselves, whilst only a modest positive correlation with NT-proBNP. Compared to renal markers urea and eGFR, the BNP molecular forms were slightly less correlated compared to NT-proBNP.

BP, blood pressure; eGFR, estimated glomerular filtration rate; Spearman's rho, r_s

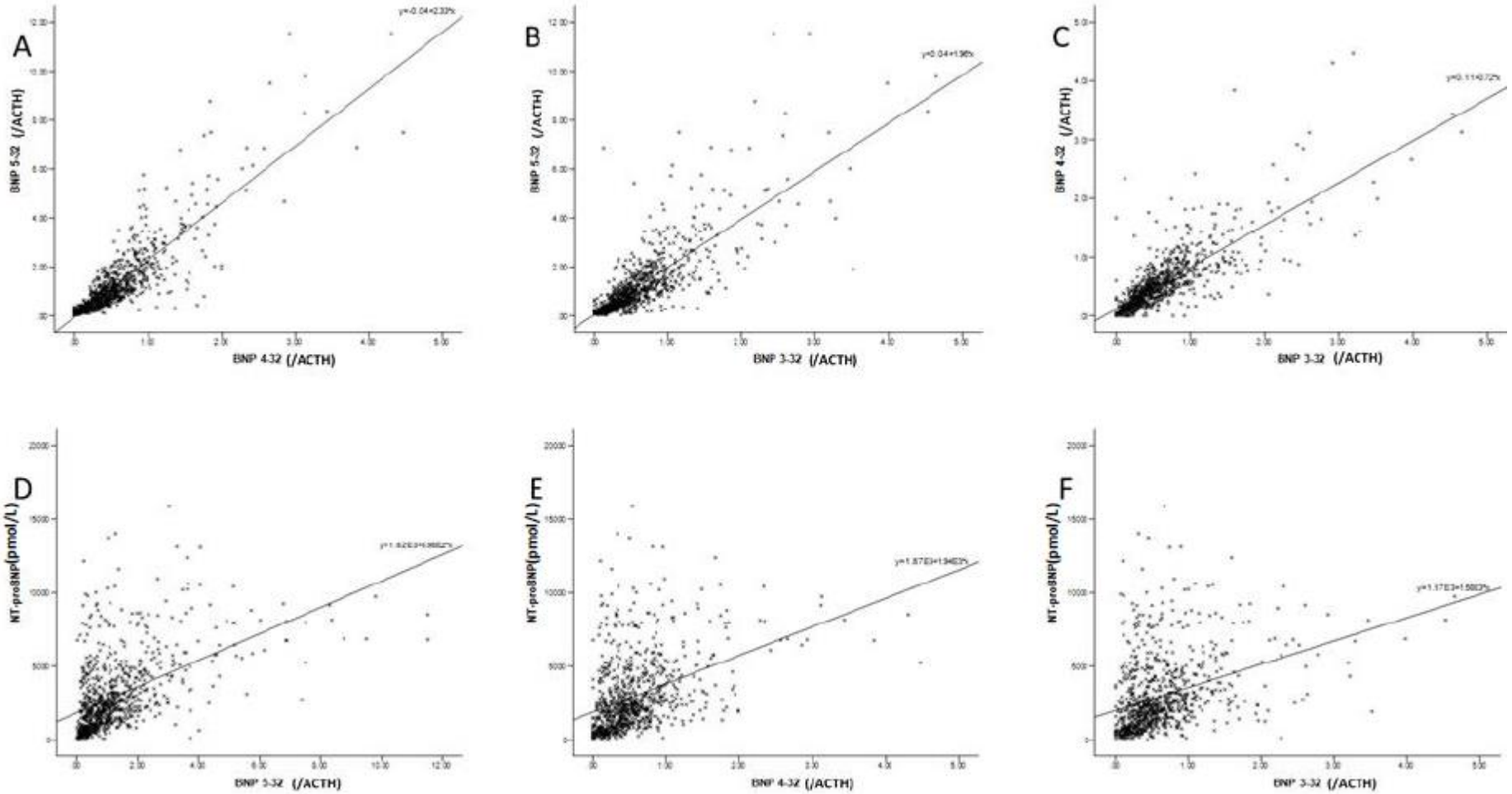


Figure 3.2 Correlation between BNP molecular forms (r_s = 0.809-0.863) and NT-proBNP (r_s = 0.438-0.557). A- BNP 5-32 and BNP 4-32, B- BNP 5-32 and BNP 3-32, C- BNP 4-32 and BNP 3-32, D- NT-proBNP and BNP 5-32, E- NT-proBNP and BNP 4-32, F- NT-proBNP and BNP 3-32

Table 3.3 Linear regression model for independent predictors of BNP molecular forms and NT-proBNP

BNP 5-32			**BNP 4-32**			**BNP 3-32**			**NT-proBNP**		
Variable	**Std β**	**p Value**	**Variable**	**Std β**	**p Value**	**Variable**	**Std β**	**p Value**	**Variable**	**Std β**	**p Value**
Age	0.128	0.002	eGFR	-0.181	0.001	NYHA Class	0.125	0.001	Urea	0.163	<0.001
PH DM	-0.110	0.004	PH DM	-0.114	0.003	eGFR	-0.153	0.005	Raised JVP	0.120	<0.001
DBP	0.125	0.008	NYHA Class	0.105	0.006	PH DM	-0.098	0.012	PH IHD	-0.134	<0.001
SBP	-0.120	0.011							Age	0.122	0.001
eGFR	-0.138	0.011							PH HF	0.096	0.007
Urea	0.123	0.019							PH DM	-0.070	0.045

The table shows that eGFR was an independent predictor for all BNP molecular forms, whereas urea was able to independently predict in NT-proBNP and BNP 5-32.Past history of DM was the only independent predictor of BNP molecular forms and NT-proBNP but not the most significant in any.

AF, atrial fibrillation; DM, diabetes; eGFR, estimated glomerular filtration rate; HTN, hypertension; IHD, ischaemic heart history; JVP, jugular venous pressure; NYHA, New York Heart Association; PH, past history; RF, renal failure; Std β, standardised beta

3.3.3 BNP molecular forms as predictors of death

To investigate prognostic ability of BNP 5-32, BNP 4-32 and BNP 3-32 for death at 6 months and at 1 year and to compare these qualities to NT-proBNP, Cox survival analyses were conducted using a multivariable base model of cardiovascular disease risk factors including; age, sex, past histories of cardiac risk markers (HF, hypertension, ischaemia, renal failure and diabetes), NYHA class, systolic blood pressure, respiratory rate, blood urea, eGFR and blood sodium. Independent abilities for the BNP molecular forms and NT-proBNP to predict outcome were tested by individually adding each biomarker to the base model. NT-proBNP was a univariate predictor of death at 6 months ($p \leq 0.001$) and 1 year ($p \leq 0.001$), and retained independent prediction for both endpoints (both $p=0.014$) when adjusted for confounding variables. Similarly, BNP 5-32, 4-32 and 3-32 were all univariate predictors of death at 6 months ($p \leq 0.001$) and at 1 year ($p \leq 0.001$). When BNP molecular forms were added to the base model, all molecular forms showed comparable predictive abilities to NT-proBNP and were able to independently predict death at 6 months ($p \leq 0.032$) and at 1 year ($p \leq 0.018$). Table 3.4 details the model statistics for all BNP measurements as predictors of death at 6 months and at 1 year. BNP 5-32 was the most superior of the molecular forms overall with a strong independent predictive ability at both 6 months [HR (95% CI) 1.31 (1.08 to 1.57), $p=0.005$] and 1 year [(HR (95% CI) 1.29 (1.10 to 1.51), $p=0.002$].

Other traditional markers that showed prediction abilities in this model at 6 months and 1 year are shown in Tables 3.5 and 3.6. Age, systolic BP, blood sodium, respiratory rate, blood urea and past history of hypertension all showed these abilities when adjusted for by each of the molecular forms and NT-proBNP ($p < 0.024$). For 1 year, age, systolic BP, blood sodium, respiratory rate, blood urea, PH hypertension and heart rate all showed these abilities when adjusted for by each of the molecular forms and NT-proBNP ($p<0.031$).

Kaplan-Meier survival analysis indicates an estimate of the survival function where a declining step is the occurrence of an event such as death. Kaplan-Meier survival analyses were performed to visualise the relationship of BNP molecular forms and death at 6 months and 1 year after stratification by tertiles. Mantel-Cox log rank tests reported significant decreases in survival across tertiles for all molecular forms at 6 months ($p \leq 0.004$) and 1 year (all $p<0.001$). BNP 5-32 and NT-proBNP showed similar survival characteristics with increased

event occurrence across higher levels, with differences observed beginning from point of admission. Alternatively, BNP 4-32 and 3-32 showed decreased survival rates across tertiles but differences were only observed between the upper 2 tertiles after approximately 3 months post-admission (Figures 3.3 and 3.4). ROC curves were comparable between molecular forms for death at 6 months (AUC 0.59 to 0.64) and 1 year (AUC 0.60 to 0.64) and are detailed in Figures 3.7 and 3.8. The AUC refers to the sensitivity/specificity balance in a risk prediction model for biomarkers into one number. For random guessing, the AUC would be 0.5 (y=x diagonal line) and would represent the worst case for a biomarker whereas AUC=1 would show perfect discrimination. The results in this analysis showing AUC 0.59-0.64 suggest average discriminatory power.

3.3.4 BNP molecular forms as predictors of death/HF

In order to investigate independent predictors of death/HF at 6 months and 1 year, Cox survival analyses were conducted employing the same base model as described for the analyses on the outcome of death. NT-proBNP was a univariate predictor of death at 6 months ($p \leq 0.001$) and 1 year ($p \leq 0.001$), and retained independent prediction for both endpoints (6 months, $p=0.035$; 1 year, $p=0.039$) after adjustment. BNP 5-32, 4-32 and 3-32 were again univariate predictors of death/HF at 6 months ($p \leq 0.001$) and at 1 year ($p \leq 0.001$) and retained independent prediction death/HF at 6 months ($p \leq 0.035$) and at 1 year ($p \leq 0.039$) in a comparable manner to NT-proBNP. Table 3.4 details the model statistics for BNP measurements as predictors of death/HF at 6 months and 1 year.

Other traditional markers that showed prediction abilities in this model of death/HF at 6 months and 1 year are shown in Tables 3.7 and 3.8. For 6 months, respiratory rate, heart rate, blood urea, blood sodium, systolic BP, and NYHA class all showed these abilities when adjusted for by each of the molecular forms and NT-proBNP ($p<0.045$). For 1 year, blood urea, heart rate, respiratory rate, systolic BP, NYHA class, PH diabetes, PH hypertension, PH HF and blood sodium all showed these abilities when adjusted for by each of the molecular forms and NT-proBNP ($p<0.049$).

Kaplan-Meier survival analyses were performed to visualise the relationship of BNP molecular forms and death/HF at 6 months and 1 year after stratification by tertiles. Mantel-Cox log rank tests reported decreases in survival across tertiles for all molecular forms at 6 months

(p≤0.003) and 1 year (p≤0.004). Similarly to survival curves for the endpoint of death, BNP 5-32 and NT-proBNP reported similar event incidence for death/HF with an increase across tertiles. However, BNP 4-32 and 3-32 reported increased but similar event occurrence for the upper 2 tertiles in comparison to the lowest tertile (Figures 3.5 and 3.6). ROC curves were comparable between molecular forms for death/HF at 6 months (AUC 0.58 to 0.60) and 1 year (AUC 0.57 to 0.60) and are detailed in Figures 3.9 and 3.10. The results in this analysis showing AUC 0.57-0.60 suggest average discriminatory power.

3.3.5 BNP molecular forms as predictors of in-hospital mortality

To investigate the usefulness of BNP 5-32, BNP 4-32 and BNP 3-32 as additive biomarkers for risk assessment of in-hospital mortality, analyses were performed alongside established clinical risk scores as either categorical (ADHERE) or continuous variables (ADHERE, OPTIMIZE-HF and GWTG-HF). BNP 5-32 was a univariate predictor of in-hospital mortality [odds ratio (OR) (95% CI) 1.45 (1.10 to 1.91, p=0.009)] as was NT-proBNP [OR 2.82 (1.64 to 4.86), p<0.001]. BNP 4-32 and 3-32, however, were not univariate predictors [BNP 4-32, OR 1.32 (0.99 to 1.75), p=0.059; BNP 3-32, OR 1.26 (0.96 to 0.67, p=0.102)]. After adjustment for clinical risk scores, BNP 5-32 was not able to independently predict in-hospital mortality (p≥0.279) nor was NT-proBNP when combined with ADHERE score [OR 1.74 (0.98 to 3.07), p=0.057] or OPTIMIZE-HF score [OR 1.67 (0.95 to 2.94), p=0.077] as a categorical variable. These results were not comparable to a previous investigation into NT-proBNP and in-hospital mortality in this cohort (Suzuki et al., 2016) as only patient samples with detectable BNP molecular forms were included in the present analysis.

Table 3.4 Independent prediction abilities of BNP molecular forms using multivariate Cox survival analyses for outcomes of death and death/HF at 6 months and 1 year

Death at 6 months

Biomarker	HR	95% CI	p Value
BNP 5-32	1.31	1.08 to 1.57	0.005
BNP 4-32	1.22	1.02 to 1.48	0.032
BNP 3-32	1.26	1.05 to 1.52	0.014
NT-proBNP	1.56	1.09 to 2.22	0.014

Death at 1 year

Biomarker	HR	95% CI	p Value
BNP 5-32	1.29	1.10 to 1.51	0.002
BNP 4-32	1.21	1.04 to 1.43	0.018
BNP 3-32	1.25	1.06 to 1.46	0.006
NT-proBNP	1.45	1.07 to 1.95	0.014

Death/HF at 6 months

Biomarker	HR	95% CI	p Value
BNP 5-32	1.18	1.02 to 1.36	0.022
BNP 4-32	1.17	1.02 to 1.35	0.031
BNP 3-32	1.21	1.05 to 1.36	0.008
NT-proBNP	1.32	1.02 to 1.72	0.035

Death/HF at 1 year

Biomarker	HR	95% CI	p Value
BNP 5-32	1.18	1.04 to 1.34	0.011
BNP 4-32	1.17	1.02 to 1.35	0.031
BNP 3-32	1.16	1.02 to 1.32	0.020
NT-proBNP	1.27	1.01 to 1.60	0.039

Models adjusted for: age, Sex, PH (Past History) Heart Failure, PH Hypertension, PH ischaemic heart disease, PH renal failure, PH diabetes, New York Heart Association class, heart rate, systolic blood pressure, respiratory rate, urea, estimated Glomerular Filtration Rate and blood sodium.

95% CI, 95% confidence intervals; HR, hazard ratio

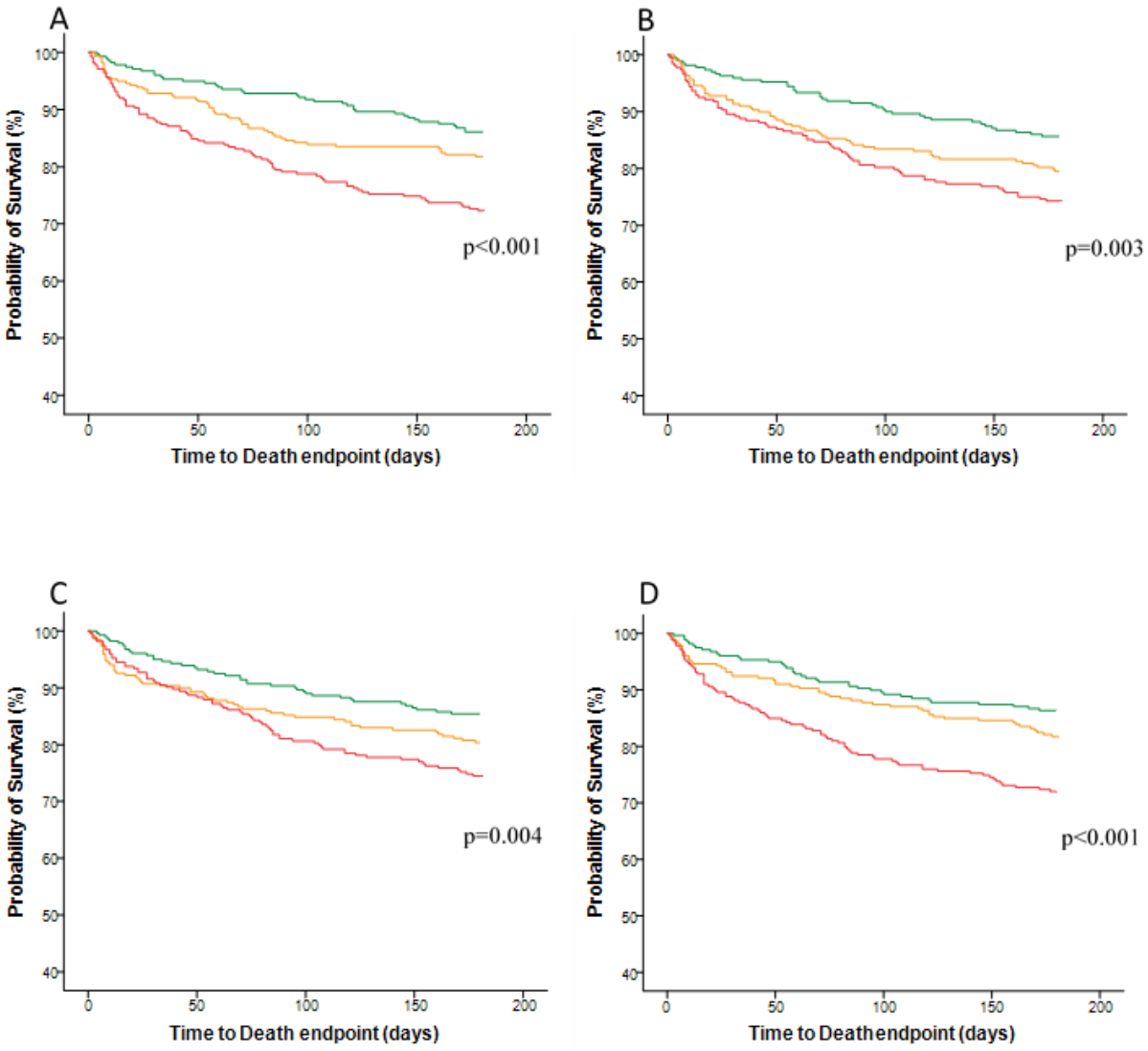


Figure 3.3 Kaplan-Meier curves showing the relationship of molecular forms of BNP and NT-proBNP and all-cause mortality (death) at 6 months after stratification by tertiles.

A- BNP 5-32, **B**- BNP 4-32, **C**- BNP 3-32, **D**- NT-proBNP

Green line – lower tertile; Orange line – middle tertile; Red line – upper tertile

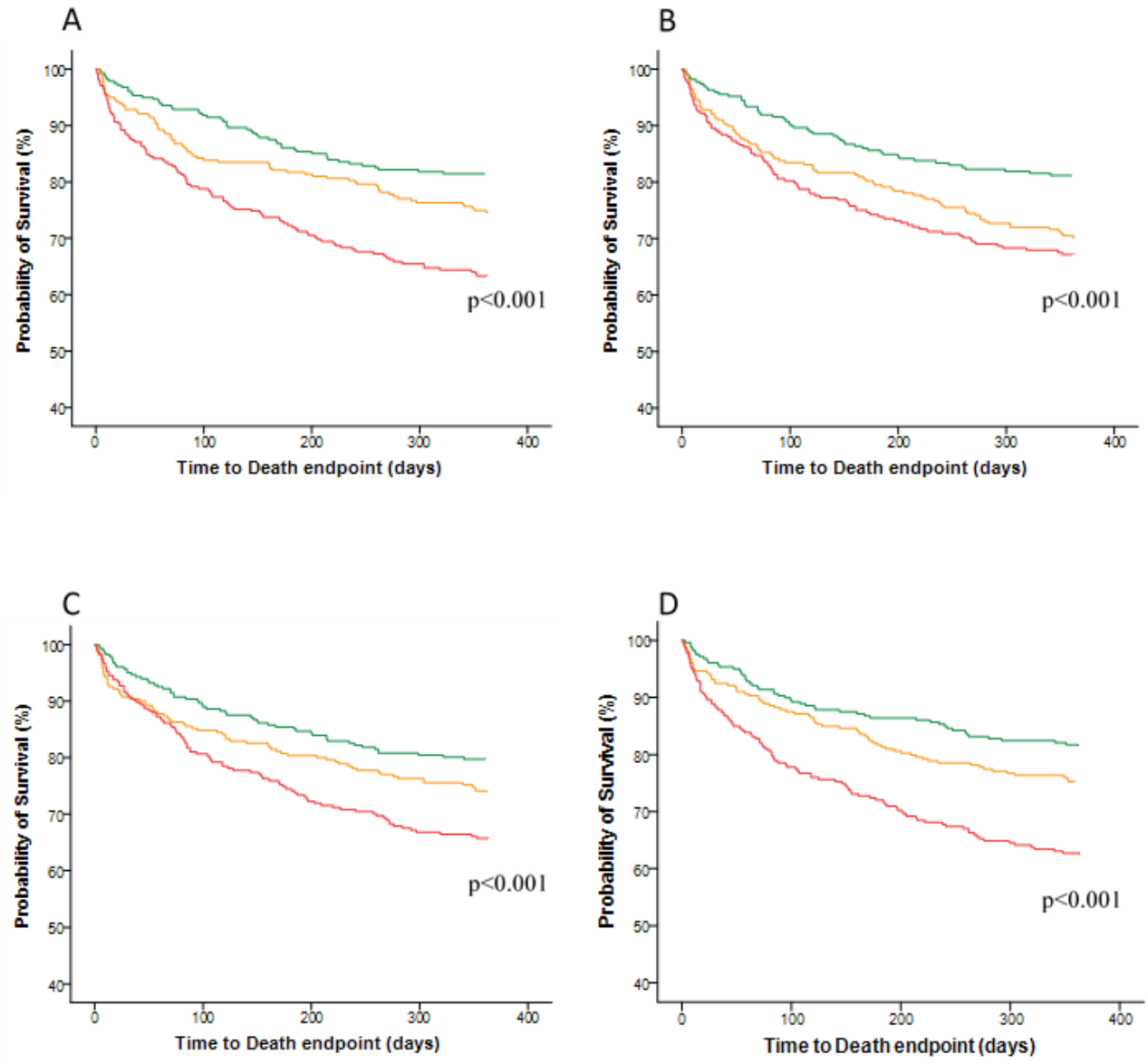


Figure 3.4 Kaplan-Meier curves showing the relationship of molecular forms of BNP and NT-proBNP and all-cause mortality (death) at 1 year after stratification by tertiles.

A- BNP 5-32, **B**- BNP 4-32, **C**- BNP 3-32, **D**- NT-proBNP

Green line – lower tertile; Orange line – middle tertile; Red line – upper tertile

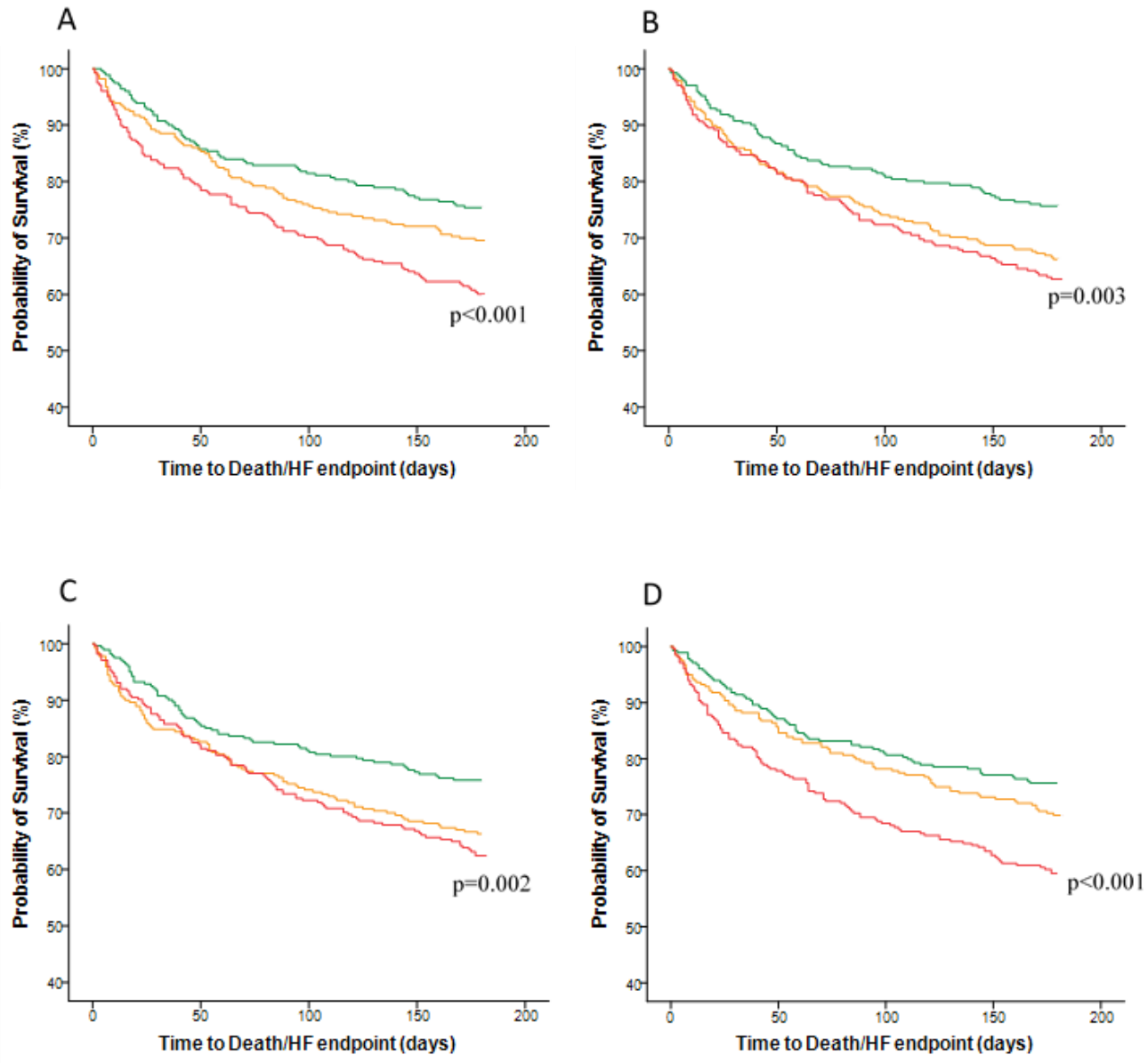


Figure 3.5 Kaplan-Meier curves showing the relationship of molecular forms of BNP and NT-proBNP and all-cause mortality or rehospitalisation due to heart failure (death/HF) at 6 months after stratification by tertiles.

A- BNP 5-32, **B**- BNP 4-32, **C**- BNP 3-32, **D**- NT-proBNP

Green line – lower tertile; Orange line – middle tertile; Red line – upper tertile

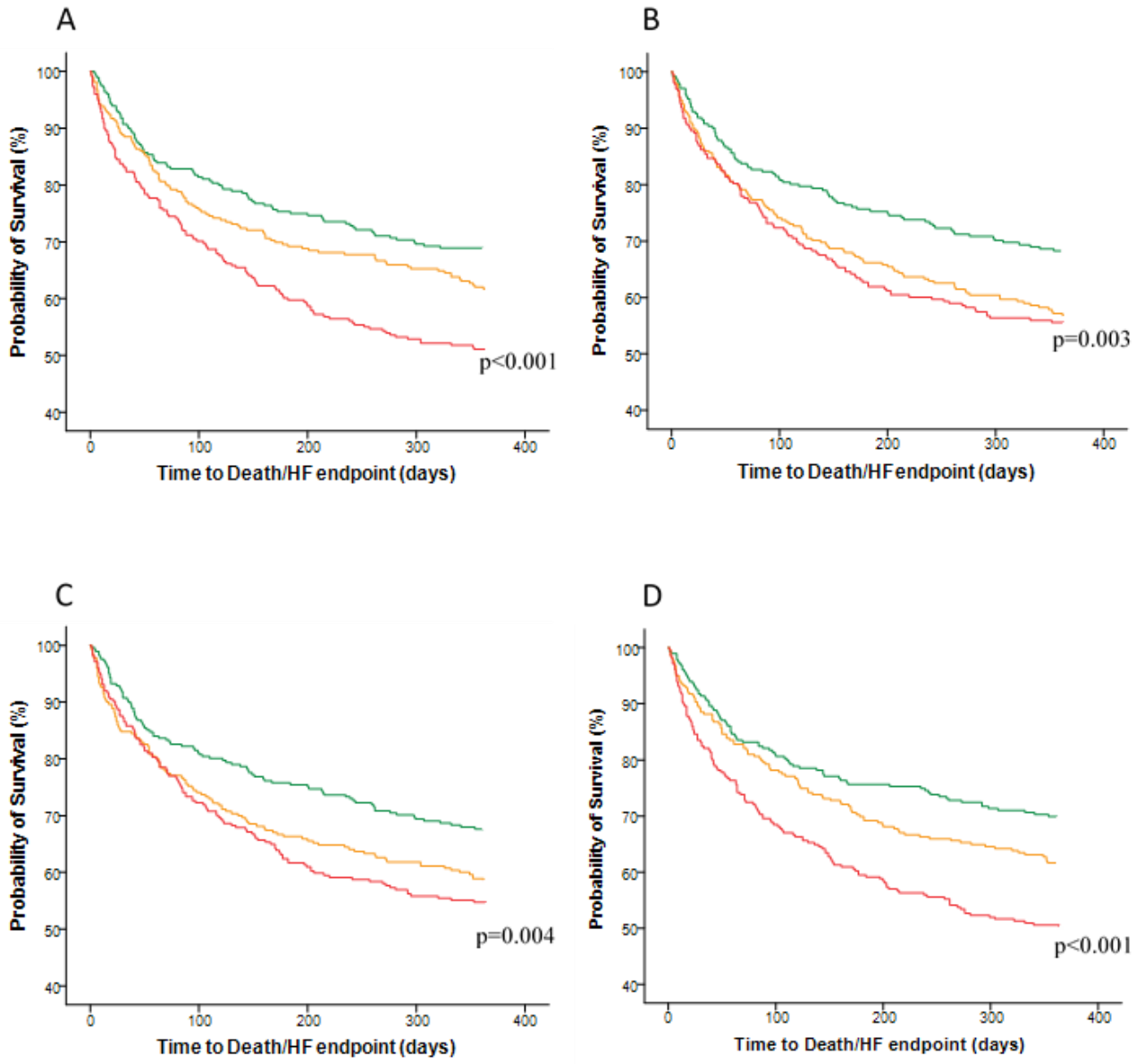
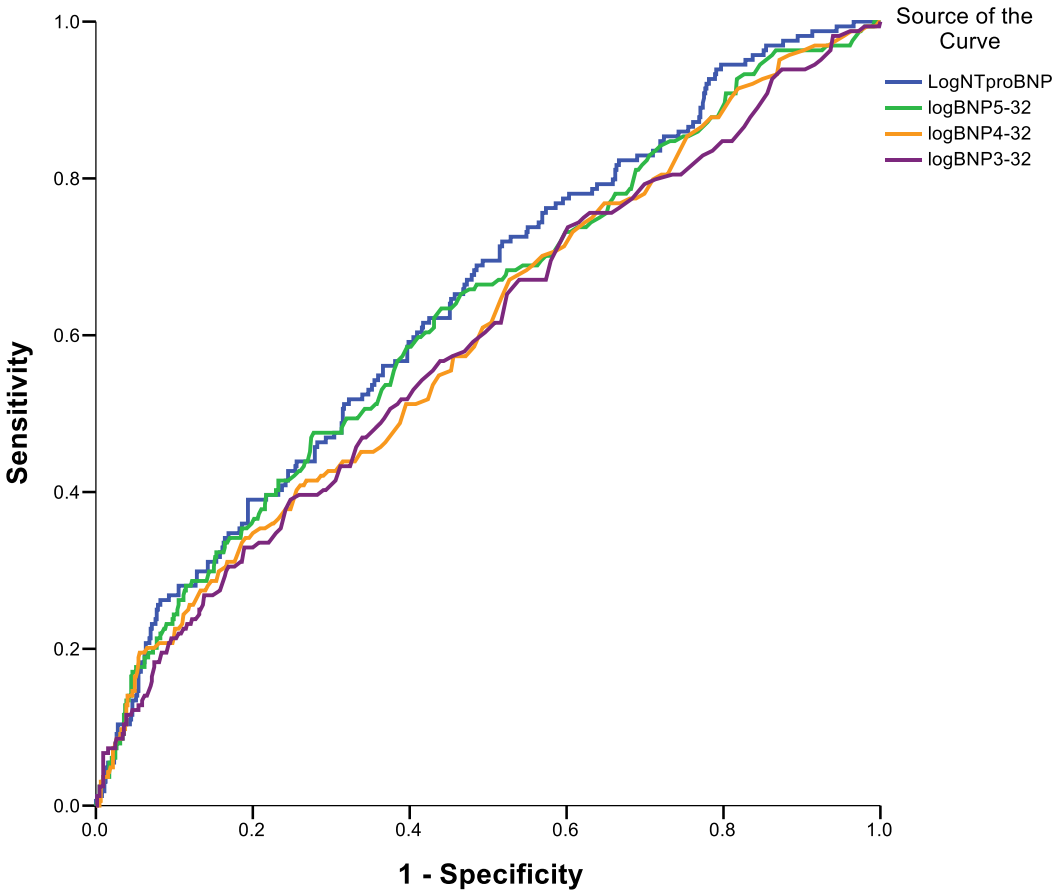


Figure 3.6 Kaplan-Meier curves showing the relationship of molecular forms of BNP and NT-proBNP and all-cause mortality or rehospitalisation due to heart failure (death/HF) at 1 year after stratification by tertiles.

A- BNP 5-32, **B**- BNP 4-32, **C**- BNP 3-32, **D**- NT-proBNP

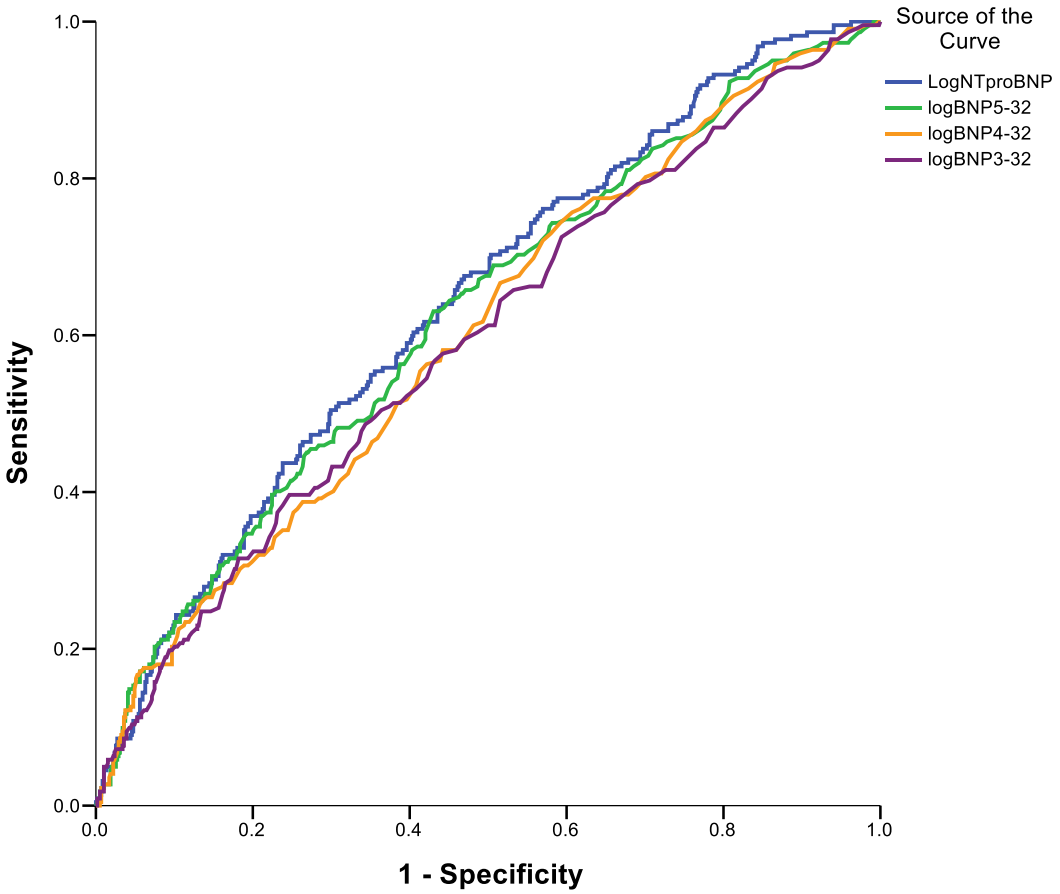
Green line – lower tertile; Orange line – middle tertile; Red line – upper tertile



Death at 6 months			
Biomarker	**AUC**	**95% CI**	**p Value**
BNP 5-32	0.63	0.58 - 0.67	<0.001
BNP 4-32	0.60	0.55 - 0.65	<0.001
BNP 3-32	0.59	0.54 - 0.64	<0.001
NT-proBNP	0.64	0.56 - 0.69	<0.001

Figure 3.7 Accuracy of outcome of prediction of log BNP molecular forms and log NT-proBNP for death at 6 months showing area under the curve (AUC) for the Receiver Operator Characteristic

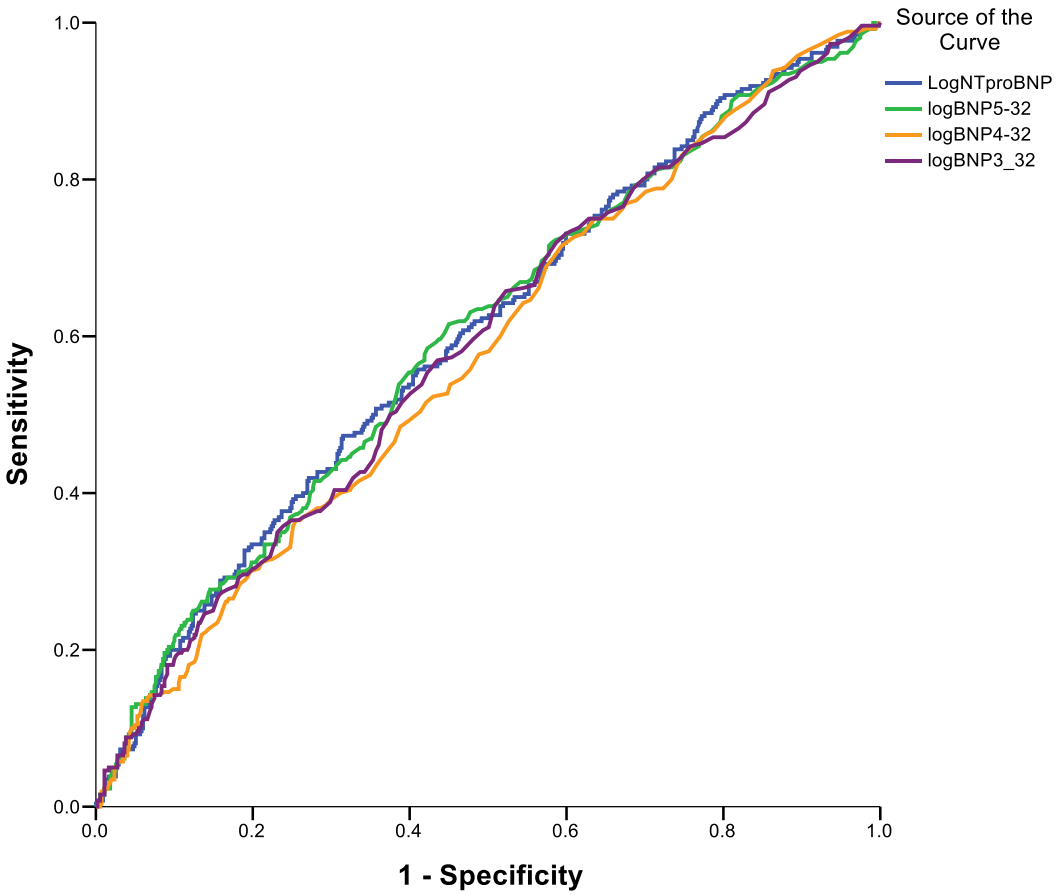
AUC, area under curve; BNP, B-type natriuretic peptide; CI, confidence interval



Death at 1 year			
Biomarker	**AUC**	**95% CI**	**p Value**
BNP 5-32	0.63	0.58 - 0.67	<0.001
BNP 4-32	0.60	0.56 - 0.65	<0.001
BNP 3-32	0.60	0.55 - 0.64	<0.001
NT-proBNP	0.64	0.60 - 0.68	<0.001

Figure 3.8 Accuracy of outcome of prediction of log BNP molecular forms and log NT-proBNP for death at 1 year showing area under the curve (AUC) for the Receiver Operator Characteristic

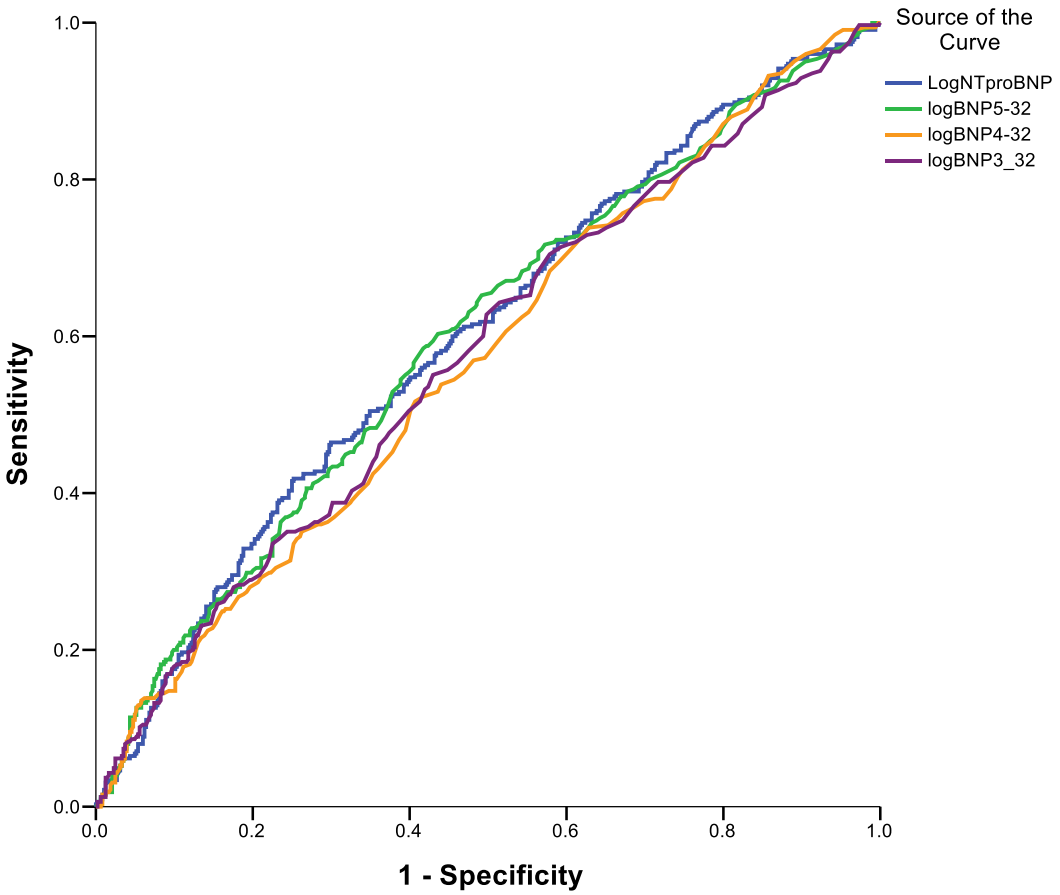
AUC, area under curve; BNP, B-type natriuretic peptide; CI, confidence interval



Death/HF at 6 months			
Biomarker	**AUC**	**95% CI**	**p Value**
BNP 5-32	0.60	0.56 - 0.64	<0.001
BNP 4-32	0.58	0.54 - 0.62	<0.001
BNP 3-32	0.59	0.54 - 0.63	<0.001
NT-proBNP	0.60	0.56 - 0.64	<0.001

Figure 3.9 Accuracy of outcome of prediction of log BNP molecular forms and log NT-proBNP for death at 6 months/HF showing area under the curve (AUC) for the Receiver Operator Characteristic

AUC, area under curve; BNP, B-type natriuretic peptide; CI, confidence interval



Death/HF at 1 year			
Biomarker	**AUC**	**95% CI**	**p Value**
BNP 5-32	0.60	0.56 - 0.64	<0.001
BNP 4-32	0.57	0.30 - 0.61	0.001
BNP 3-32	0.58	0.54 - 0.62	<0.001
NT-proBNP	0.60	0.56 - 0.64	<0.001

Figure 3.10 Accuracy of outcome of prediction of log BNP molecular forms and log NT-proBNP for death at 1 year/HF showing area under the curve (AUC) for the Receiver Operator Characteristic

AUC, area under curve; BNP, B-type natriuretic peptide; CI, confidence interval

Table 3.5 Independent prediction abilities of traditional cardiac risk markers using multivariate Cox survival analyses for outcomes of all-cause mortality at 6 months in models including molecular forms of BNP or NT-proBNP.

BNP 5-32			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 1.00	<0.001
Blood sodium	0.94	0.92 to 0.97	<0.001
Respiratory rate	1.05	1.02 to 1.08	0.001
PH HTN	0.63	0.44 to 0.89	0.009
Blood Urea	1.04	1.01 to 1.07	0.015
PH DM	1.50	1.03 to 2.18	0.035

BNP 4-32			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 1.00	<0.001
Blood sodium	0.95	0.92 to 0.97	<0.001
Respiratory rate	1.05	1.02 to 1.08	0.001
PH HTN	0.60	0.42 to 0.86	0.005
Blood Urea	1.04	1.01 to 1.07	0.011
PH DM	1.46	1.00 to 2.14	0.048

BNP 3-32			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.98	0.98 to 0.99	<0.001
Blood sodium	0.95	0.92 to 0.97	<0.001
Respiratory rate	1.05	1.02 to 1.08	0.002
Blood Urea	1.04	1.01 to 1.07	0.010
PH HTN	0.66	0.46 to 0.95	0.024

NT-proBNP			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 0.99	<0.001
Blood sodium	0.94	0.92 to 0.97	<0.001
Respiratory rate	1.05	1.02 to 1.07	0.001
PH HTN	0.65	0.46 to 0.92	0.017
Blood Urea	1.04	1.01 to 1.07	0.019
PH DM	1.46	1.00 to 2.13	0.046

Models adjusted for: age, sex, PH (past history) heart failure, PH hypertension, PH ischaemic heart disease, PH renal failure, PH diabetes, New York Heart Association class, heart rate, systolic blood pressure, respiratory rate, blood urea, estimated glomerular filtration rate and blood sodium

95% CI, 95% confidence intervals; BP, blood pressure; DM, diabetes; HTN, hypertension; HR, hazard ratio

Table 3.6 Independent prediction abilities of traditional cardiac risk markers using multivariate Cox survival analyses for outcomes of all-cause mortality at 1 year in models including molecular forms of BNP or NT-proBNP.

BNP 5-32			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 0.99	<0.001
Blood Urea	1.05	1.02 to 1.07	<0.001
Blood sodium	0.95	0.92 to 0.97	<0.001
Respiratory rate	1.04	1.02 to 1.07	0.001
PH HTN	0.65	0.48 to 0.88	0.005
Heart rate	0.99	0.99 to 1.00	0.018
NYHA class	1.35	1.02 to 1.79	0.034

BNP 4-32			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 0.99	<0.001
Blood Urea	1.05	1.02 to 1.07	<0.001
Blood sodium	0.95	0.93 to 0.98	<0.001
Respiratory rate	1.05	1.02 to 1.07	0.001
PH HTN	0.63	0.47 to 0.86	0.004
Heart rate	0.99	0.99 to 1.00	0.020
NYHA class	1.37	1.03 to 1.82	0.031

BNP 3-32			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 0.99	<0.001
Blood Urea	1.05	1.02 to 1.07	<0.001
Blood sodium	0.95	0.93 to 0.97	<0.001
Respiratory rate	1.04	1.02 to 1.07	0.002
PH HTN	0.68	0.50 to 0.99	0.013
Heart rate	0.99	0.99 to 1.00	0.023

NT-proBNP			
Biomarker	**HR**	**95% CI**	**p Value**
Age	1.04	1.02 to 1.06	<0.001
Systolic BP	0.99	0.98 to 0.99	<0.001
Blood sodium	0.95	0.93 to 0.97	<0.001
Blood Urea	1.04	1.02 to 1.07	0.001
Respiratory rate	1.04	1.02 to 1.07	0.001
PH HTN	0.67	0.49 to 0.90	0.009
Heart rate	0.99	0.99 to 1.00	0.031
NYHA class	1.36	1.03 to 1.80	0.034

Models adjusted for: age, sex, PH (past history) heart failure, PH hypertension, PH ischaemic heart disease, PH renal failure, PH diabetes, New York Heart Association class, heart rate, systolic blood pressure, respiratory rate, blood urea, estimated glomerular filtration rate and blood sodium

95% CI, 95% confidence intervals; BP, blood pressure; DM, diabetes; HTN, hypertension; HR, hazard ratio; NYHA, New York Heart Association

Table 3.7 Independent prediction abilities of traditional cardiac risk markers using multivariate Cox survival analyses for outcomes of all-cause mortality or rehospitalisation due to heart failure at 6 months in models including molecular forms of BNP or NT-proBNP.

BNP 5-32			
Biomarker	**HR**	**95% CI**	**p Value**
Respiratory rate	1.03	1.01 to 1.06	0.004
Heart rate	0.99	0.99 to 1.00	0.005
Blood Urea	1.03	1.01 to 1.06	0.008
Blood sodium	0.97	0.95 to 0.99	0.011
Systolic BP	0.99	0.99 to 1.00	0.021
NYHA class	1.33	1.04 to 1.71	0.024
PH HTN	0.73	0.55 to 0.96	0.026
PH DM	1.38	1.04 to 1.85	0.028

BNP 4-32			
Biomarker	**HR**	**95% CI**	**p Value**
Respiratory rate	1.04	1.01 to 1.06	0.002
Heart rate	0.99	0.99 to 1.00	0.003
Blood Urea	1.03	1.01 to 1.06	0.009
Systolic BP	0.99	0.99 to 1.00	0.014
Blood sodium	0.97	0.95 to 1.00	0.017
PH HTN	0.71	0.54 to 0.94	0.018
PH DM	1.40	1.04 to 1.85	0.031
NYHA class	1.31	1.02 to 1.69	0.038

BNP 3-32			
Biomarker	**HR**	**95% CI**	**p Value**
Heart rate	0.92	0.99 to 1.00	0.005
Blood Urea	1.03	1.01 to 1.06	0.006
Respiratory rate	1.31	1.01 to 1.06	0.007
Systolic BP	0.99	0.99 to 1.00	0.013
Blood sodium	0.97	0.95 to 1.00	0.017
NYHA class	1.23	1.01 to 1.66	0.045

NT-proBNP			
Biomarker	**HR**	**95% CI**	**p Value**
Respiratory rate	1.03	1.01 to 1.05	0.004
Heart rate	0.99	0.99 to 1.00	0.007
Blood Urea	1.03	1.01 to 1.06	0.009
Blood sodium	0.97	0.95 to 0.99	0.010
NYHA class	1.33	1.04 to 1.71	0.025
Systolic BP	0.99	0.99 to 1.00	0.028
PH DM	1.36	1.02 to 1.81	0.037
PH HTN	0.75	0.57 to 0.99	0.040

Models adjusted for: age, sex, PH (past history) heart failure, PH hypertension, PH ischaemic heart disease, PH renal failure, PH diabetes, New York Heart Association class, heart rate, systolic blood pressure, respiratory rate, blood urea, estimated glomerular filtration rate and blood sodium

95% CI, 95% confidence intervals; BP, blood pressure; DM, diabetes; HTN, hypertension; HR, hazard ratio; NYHA, New York Heart Association

Table 3.8 Independent prediction abilities of traditional cardiac risk markers using multivariate Cox survival analyses for outcomes of all-cause mortality or rehospitalisation due to heart failure at 1 year in models including molecular forms of BNP or NT-proBNP.

BNP 5-32			
Biomarker	**HR**	**95% CI**	**p Value**
Blood Urea	1.04	1.02 to 1.06	<0.001
Heart rate	0.99	0.99 to 1.00	0.002
Respiratory rate	1.03	1.01 to 1.05	0.003
Systolic BP	0.99	0.99 to 1.00	0.012
NYHA class	1.32	1.06 to 1.65	0.015
PH DM	1.36	1.05 to 1.76	0.022
PH HTN	0.75	0.58 to 0.96	0.023
PH HF	1.33	1.03 to 1.73	0.030
Blood sodium	0.98	0.96 to 1.00	0.032

BNP 4-32			
Biomarker	**HR**	**95% CI**	**p Value**
Blood Urea	1.04	1.02 to 1.06	<0.001
Heart rate	0.99	0.99 to 1.00	0.001
Respiratory rate	1.04	1.01 to 1.06	0.002
Systolic BP	0.99	0.99 to 1.00	0.008
PH HTN	0.73	0.57 to 0.94	0.015
PH HF	1.36	1.05 to 1.77	0.022
NYHA class	1.30	1.04 to 1.64	0.023
PH DM	1.35	1.04 to 1.76	0.024
Blood sodium	0.99	0.96 to 1.00	0.043

BNP 3-32			
Biomarker	**HR**	**95% CI**	**p Value**
Blood Urea	1.04	1.02 to 1.06	<0.001
Heart rate	0.99	0.99 to 1.00	0.002
Respiratory rate	1.03	1.01 to 1.05	0.005
Systolic BP	0.99	0.99 to 1.00	0.007
NYHA class	1.30	1.03 to 1.62	0.024
PH HF	1.33	1.03 to 1.73	0.031
Blood sodium	0.98	0.96 to 1.00	0.046
PH DM	1.30	1.00 to 1.69	0.048
PH HTN	0.78	0.61 to 1.00	0.049

NT-proBNP			
Biomarker	**HR**	**95% CI**	**p Value**
Blood Urea	1.04	1.02 to 1.06	0.001
Heart rate	0.99	0.99 to 1.00	0.003
Respiratory rate	1.03	1.01 to 1.05	0.003
NYHA class	1.32	1.06 to 1.65	0.014
Systolic BP	0.99	0.99 to 1.00	0.016
Blood sodium	0.98	0.96 to 1.00	0.030
PH DM	1.33	1.03 to 1.73	0.031
PH HTN	0.76	0.60 to 0.98	0.034
PH HF	1.32	1.02 to 1.72	0.035

Models adjusted for: age, sex, PH (past history) heart failure, PH hypertension, PH ischaemic heart disease, PH renal failure, PH diabetes, New York Heart Association class, heart rate, systolic blood pressure, respiratory rate, blood urea, estimated glomerular filtration rate and blood sodium

95% CI, 95% confidence intervals; BP, blood pressure; DM, diabetes; HF, heart failure; HTN, hypertension; HR, hazard ratio; NYHA, New York Heart Association

3.4 Discussion

This study reports that BNP molecular forms are associated with a prognosis of poor outcome in patients hospitalised with acute HF. Molecular forms of BNP 5-32, 4-32 and 3-32 were all independently able to predict all-cause mortality or a combination of all-cause mortality and rehospitalisation due to HF at 6 months and 1 year after adjustment for a range of clinical and physiological factors. When stratified by tertiles, BNP molecular forms showed increased risk of adverse event with elevated circulating levels. Previous reports have looked at the association of BNP molecular forms with heart failure, however, this study is the first prognostic investigation into processed BNP forms in acute HF (Fujimoto et al., 2013, Niederkofler et al., 2008, Miller et al., 2011).

BNP 5-32 displayed to be the superior molecular form for the prediction of death and/or death/HF events using proportional hazard analyses. In addition to this, when categorised into tertiles, BNP 5-32 was observed to show the strongest association between elevated levels and reduced survival. The reasons for the slight increase in association seen for BNP 5-32 over 4-32 and 3-32 is not known but may be linked to the kinetics and dynamics of BNP fragmentation characteristics. However, BNP molecular forms were not additive for the risk prediction of in-hospital mortality when combined with current clinical risk scores. This suggesting at a short period of time, such as in-hospital mortality, NT-proBNP is released into the blood whereas BNP molecular forms are degraded forms of BNP-32 and therefore require longer processing time (Brandt et al., 2006). Although BNP molecular forms have not been previously investigated, BNP-32 has however been shown to be a strong prognostic indicator for HF patients at all stages of disease. Data from five studies also showed that an increase of 100 pg/mL in BNP caused a 35% increased risk of death (Doust et al., 2005).

Prognostic data obtained for the measured BNP molecular forms was investigated alongside measurements of NT-proBNP and were observed to be favourable for prediction of death or death/HF at 6 months and 1 year. Investigations into associations with renal markers showed that all molecular forms were significantly correlated with blood urea and eGFR, but at a level that was modestly reduced when compared with NT-proBNP (Table 3.2). As renal dysfunction is a common morbidity associated with HF,

and approximately 55-60% of NT-proBNP is known to be cleared by the kidneys whereas BNP molecular forms are removed through NP receptors or NEP, processed BNP molecular forms are suggested to merit for risk stratification in patients irrespective of renal function (Srisawasdi et al., 2010, Palmer et al., 2009). It is currently unknown how BNP molecular forms are affected in patients with chronic kidney disease and would benefit through further investigation.

BNP-32, the form present prior to fragmentation, is known to exhibit a short half-life of approximately 20 minutes when present in circulating blood (Potter, 2011) and processed BNP forms, such as those tested in this study, are thought to be concurrently measured in commercial testing kits (Heublein et al., 2007). This presents a suggestive role for the processed forms of BNP that is supported, particularly for BNP 5-32 and 3-32, by the data described from this population experiment. BNP levels have been observed to show time variations, with plasma levels shown to vary from day-to-day such that it is suggested to take serial measurements before reaching conclusions (Wu and Smith, 2004). For this experiment, sample collections were collected during the acute phase of HF but were not strictly time-controlled due to emergency procedures associated with admission to hospital. Earlier BNP measurements have been shown to possess reduced prognostic ability when compared to measurements recorded up to 7 days post-admission (Mega et al., 2004). With the characteristics of peptide fragmentation currently unknown, serial sampling and measurement of fragmentation profiles may offer improved information for risk stratification of acute HF admissions.

Currently, various prognostic markers are employed for death and/or HF hospitalisation in HF patients, however risk stratification remains a challenge. Multivariate models and risk scores are often used for HF patients and have shown to be able to predict death but are less useful for predicting HF hospitalisation (Ponikowski et al., 2016). In heart failure, natriuretic peptide levels increase with the severity of HF in order to fulfil the demand of the heart and reduce blood pressure (Kasahara et al., 2000). These results suggest the role for molecular forms of BNP, especially BNP 5-32, in the prognosis of acute HF. Although the precise mechanisms of these molecular forms are not fully understood, due to the prognostic role of BNP molecular forms in acute HF, investigations into the processing enzymes of the molecular forms that mediate the

actions and proportions of each molecular form and could explain their involvement in the prognosis of acute HF.

3.5 Conclusion

In conclusion, this study reports the first prognostic investigation of BNP molecular forms in a cohort of heart failure patients and describes the prognostic ability of BNP molecular forms in comparison to current clinical BNP measurement techniques. Further, this is the first report on the prognostic qualities of NT-proBNP in acute HF for events occurring at 6 months and 1 year post-initial hospital admission. Circulating plasma levels of BNP 5-32, BNP 4-32 and BNP 3-32 were associated with a prognosis of poor outcome at 6 months and 1 year in patients with acute HF, and were comparable in prognostic ability when compared with NT-proBNP.

CHAPTER FOUR

CHRONIC HEART FAILURE STUDY

4. CHRONIC HEART FAILURE STUDY (BIOSTAT-CHF)

4.1 Introduction

4.1.1 Introduction and Rationale

Both BNP and NT-proBNP have diagnostic and prognostic value in HF where decreases in their concentrations during follow-up are associated with improved outcomes, and increasing levels identify those most at risk (Savarese et al., 2014). Furthermore, many trials evaluating changes in natriuretic peptide levels from baseline and its effects on treatment monitoring have yielded mixed results due to multiple reasons including small sample size, low event rate and length of follow-up (Felker et al., 2009, Troughton et al., 2000). To extend upon baseline measurements of natriuretic peptides, the Valsartan Heart Failure Trial (Val-HeFT) observed relationships between circulating NT-proBNP levels over a period of 4 months, and associations with adverse outcomes. It was shown that serial measurements of NT-proBNP was useful for risk stratification in stable and chronic HF (Masson et al., 2008). More recently, serial NT-proBNP measurements were used to assess the changes with adverse events and the influence dependency on therapy, focussing on the new drug; sacubitril/valsartan (Entresto™). It was shown the benefits of serial measurements in HF risk management and the 1-month reduction was associated with improved outcome where the therapy was able to improve this reduction (Zile et al., 2016, Israr et al., 2017). However, the effects of BNP molecular form concentrations on treatment, serial sampling, and outcomes are currently unknown.

The systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF) was a large European multicentre, prospective, observational study from 69 centres in 11 countries (Voors et al., 2016), to characterise biological pathways related to response to therapy for HF.

4.1.2 Aims and Hypothesis

• Investigate the associations of BNP molecular forms in patients admitted with HF and followed-up at visit 2

• Assess the implications of BNP molecular forms on serial sampling and the effects of treatment in risk prediction

• Assess the applications of these measurements for use in prognostic risk prediction

Using the developed MALDI-ToF-MS immunoassay method BNP molecular forms are expected to be detected in patient plasma and show similar prognostic abilities as the clinically measured biomarker, BNP. BNP molecular forms are also hypothesised to show less detection at visit 2 displaying a correlation with the improvement in a patient's condition. BNP molecular forms are likely to be affected by treatment in a similar manner to BNP, and BNP 5-32 is expected to be the dominating molecular form.

4.2 Methods

4.2.1 Study Population

BIOSTAT-CHF study design has been previously described (Voors et al., 2016). Briefly, patients were enrolled between 2010-2014 with progressive worsening or new-onset symptoms of HF, confirmed by either left ventricular EF of ≤40% or BNP and/or NT-proBNP plasma concentrations >400pg/ml or >2000pg/ml, respectively. The BIOSTAT-CHF project itself aimed to identify the effects and response to the up-titration of medical therapy, therefore all patients underwent treatment with furosemide ≥40mg/day or equivalent and received ≤50% of target doses of angiotensin converting enzyme inhibitor (ACEi)/angiotensin receptor blockers (ARBs) and beta-blockers at time of study entry. Each patient consented to have blood samples taken and outcomes surveyed. This study was approved by the local ethics committee and adhered to the Declaration of Helsinki.

Primary endpoints were all-cause mortality, with secondary endpoints as the composite mortality with rehospitalisation due to HF (mortality/HF).

The aim of the initial cohort was to use a systems biology approach to evaluate patients with poor clinical outcome despite evidenced-based treatment. The major advantage of this study was the vast number of clinical, proteomics and biomarker data available for comparative analysis. These data was collected in 13 countries and comprised 1884 samples in total (enrolment and follow up) making this a large sample size and multi-centre (multi-Europe) study. For BNP molecular forms, the data present resembled the heterogeneity of the European patient population. Secondly, because this study collected samples from patients pre- and post-treatment, it allowed for a study to investigate the impact of serial sampling and the impact of treatment on BNP molecular form levels. BNP data was available for both visits alongside clinical data allowing for a more in-depth comparative analysis with BNP molecular forms. This cohort is a large cohort with samples collected more recently (2010-2014) indicating that the BNP peptides present are suggested to be better preserved.

However, there are limitations to this study, some of them directly related to the design of the study such as the inclusion of HFpEF despite ACE inhibitors, ARBs, and beta-blockers are not guideline-recommended therapies in patients with HFpEF (Voors et al., 2017). Furthermore, the BIOSTAT-HF is a prospective, non-randomized observational study, therefore it is not possible to infer causality to our findings or provide a mechanistic explanation. Due to heterogeneity and cultural differences there remains a possibility that other measured/unmeasured confounding factors could influence the association between BNP molecular forms and HF. Despite the advantage of a large European-wide study, all data used for this study are derived from European centres only, and 99% of patients were Caucasian, therefore the findings of this study may not be representative of HF patients at the global levels. For this study, samples were collected at multi-centres where sample collection, storage and transport could differ and would be problematic. Also, as evidenced by the low detection rate in the cohort, the patients enrolled were sub-acute/chronic and retrospectively the use of BNP molecular forms does not seem to be suited to chronic HF. Further, samples were collected only in EDTA, with the absence of a protease inhibitor (unlike the acute HF and acute MI studies), this could have been responsible for a possible degradation over time from sample collection to storage and transport.

4.2.2 Sample Collection

Patient blood samples were collected at initial enrolment (V1) and at follow-up (V2), approximately nine months apart.

4.2.3 BNP molecular form measurement

BNP molecular forms were measured and the results were compared to BNP values and further clinical markers measured either at a local hospital site or within the BIOSTAT-CHF central laboratory (Voors et al., 2016). Inter-assay relative standard deviations for daily control experiments across the course of the study for synthetic BNP 5-32, BNP 4-32 and BNP 3-32 were 5.2%, 9.8% and 14.9%, respectively. RSD was controlled within an average of 10% for the molecular forms, as shown in Figure 4.1.

4.2.4 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (V24, IBM Corp., Armonk, New York, USA). Analyses were performed using the BIOSTAT-CHF database, and modifications made where sufficient data were not available. For patients in both visits, changes in demographics from visit 1 were compared using Wilcoxon matched-pair signed-rank test for continuous variables and the McNemar test for categorical variables. Correlations between BNP molecular forms and other clinical variables were performed using Spearman's correlations. Multivariate and univariate models were used to evaluate the associations between BNP molecular forms with outcomes at 1 year, 2 years and 3 years, using Cox proportional hazard regression analyses. Compact and extended risk models were made from the previously defined BIOSTAT-CHF model (Voors et al., 2017). BNP molecular form and BNP values were log transformed.

To investigate the changes in BNP 5-32 with treatment outcomes, and the changes between visit 1 and visit 2, data was classified into four groups; no detection/no detection, no detection/detection, detection/no detection, and detection/detection. For BNP, the data was split by the median and classified by high or low relative to the median value for visit 1; low/low, low/high, high/low, and high/high. Cox proportional hazards analysis was performed to investigate the changes with outcomes, with no detection/no detection (in both visits) for BNP 5-32 and low/low in both visits (<median visit 1) for BNP assumed as the reference value. Kaplan-Meier survival curves were generated to evaluate cumulative incidence between BNP molecular forms and outcome using the Mantel-Cox log rank test after stratification by the median.

Decision tree analysis was performed using the χ^2 automatic interaction detection (CHAID) and groupings tested with outcomes using Cox proportional hazards regression and Kaplan-Meier survival curves. A p-value <0.05 was considered statistically significant.

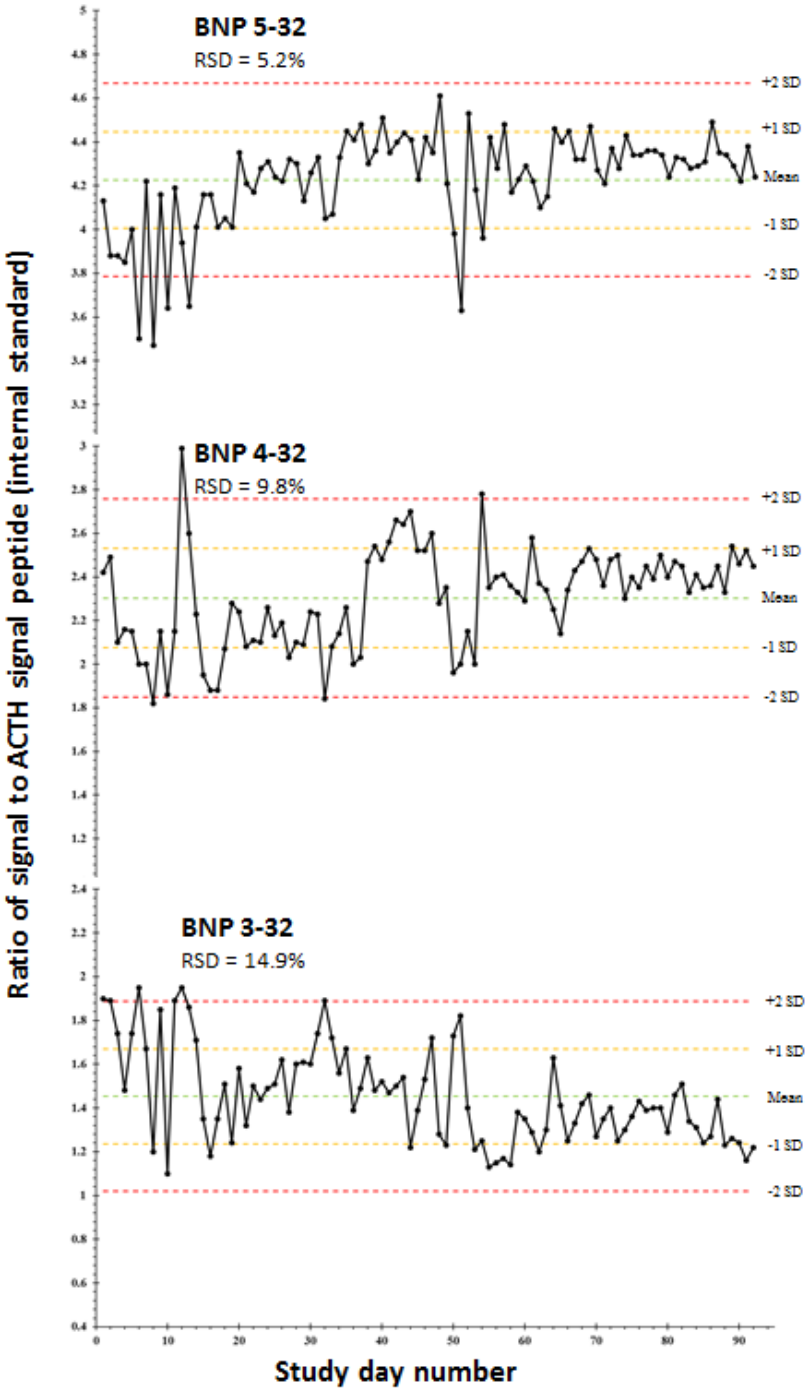


Figure 4.1 Relative Standard Deviation for synthetic BNP 5-32, BNP 4-32, and BNP 3-32 used as the positive control throughout the course of the study. Average RSD was less than the 10% limit and within three SD.

ACTH, Adrenocorticotrophic hormone; BNP, B-type natriuretic peptide; RSD, Relative standard deviation

4.3 Results

4.3.1 Cohort characteristics and demographics

Plasma from 1127 visit 1 patients and 757 visit 2 follow-up patients were analysed for the presence of BNP molecular forms. Mass spectral peaks for BNP molecular forms were detectable in a total of 761 samples (40%), 574 in visit 1 (51%) and 187 in visit 2 (25%). Analysis of trends showed that the majority of patients were male (76%) with a median age of 69 years and 29% with history of a previous hospitalisation. The majority of patients were in NYHA functional class III-IV (57%) and had elevated BNP plasma concentrations (202 pg/mL, median). A subset of 757 patients were analysed for comparisons between enrolment (V1) and follow-up (V2) approximately nine months after enrolment, and had undergone guideline-based therapy for HF. Clinical demographics showed that their clinical status improved, evidenced by a decrease in BNP concentrations (202 pg/ml, V1; 134 pg/mL, V2 $p<0.001$), decrease in pulmonary congestion (- 38%, $p<0.001$) and peripheral oedema. NYHA class also improved with the majority of patients from class III and IV at enrolment (combined 55%) to class I and II at follow-up (combined 75%). Use of beta-blocker and ACEi also increased between V1 and V2 (+8% and +15% respectively, $p<0.001$) A full breakdown of demographics can be found in Table 4.1.

4.3.2 BNP molecular forms and associated clinical measurements

The data from both visits (n=1884) for BNP molecular forms were combined to analyse the levels of correlation between the molecular forms and BNP. BNP molecular forms were strongly correlated to each other (r_s = 0.838-0.901, $p<0.001$) and also to BNP (r_s = 0.674-0.787, $p<0.001$). The levels of correlation can be observed in Figure 4.2. To analyse the correlations with other clinical variables and change from baseline, visit 1 and visit 2 data were split accordingly. These results can be found in Table 4.2. Urea (r_s = 0.116-0.324, $p<0.001$), eGFR (r_s = -0.159 - -0.379, $p<0.001$) and systolic BP (r_s = -0.118- -0.142, $p<0.001$) showed significant correlation with BNP molecular forms and BNP in both visits. Age showed significant correlation with BNP 5-32, BNP 4-32 and BNP at visit 1 and all molecular forms at visit 2. Interestingly diastolic BP was not significantly correlated to BNP molecular forms at visit 1 ($p\geq0.115$) but was significantly correlated

at visit 2 (r_s = -0.099- -0.138, p<0.001). BNP was significantly correlated in both visits (r_s = -0.082- -0.203, p≤0.006). An opposite trend was observed with blood sodium showing significant correlation at visit 1 for BNP molecular forms (r_s = -0.064- -0.088, p≤0.043), but correlations at visit 2 (p≥0.103). BNP was not significantly correlated in both visits. Similarly, heart rate was significantly correlated to BNP molecular forms and BNP in visit 1 (r_s = 0.138- 0.175, p<0.001), but was not significantly correlated in BNP molecular forms in visit 2 (p≥0.354), and negatively correlated to BNP (r_s = -0.095, p<0.001).

Furthermore, due to the high frequency of non-detectable molecular forms shown in Figure 4.2 as vertical/horizontal lines, investigations were made to assess the impact on the correlation. Results showed that when BNP 4-32 and BNP 3-32 were only considered by their detection ((/ACTH)>0), the correlations displayed against BNP and the molecular forms showed a minute, subtle difference. However, when BNP 5-32 was considered by ((/ACTH)>0), a more profound difference was observed with associations with BNP 4-32 and BNP 3-32 compared to the inclusion of the full cohort, but not a significant difference for correlations with BNP. These observations are shown in Table 4.3.

Table 4.1 Patient demographics BIOSTAT-CHF cohort

	Total cohort (n=1127)	Patients with follow-up visit (n=757)		
		Visit 1	Visit 2	p value
Age	69 (60–77)	69 (60–77)		
Male	76%	76%		
Current smoker	14%	14%		
Ischemic etiology	53%	54%		
Diabetes mellitus	31%	31%		
COPD	18%	18%		
Previous HF hospitalisation	29%	29%		
NYHA class I	3%	3%	16%	<0.001*
II	40%	47%	59%	
III	47%	47%	24%	
IV	10%	8%	1%	
LV ejection fraction (%)	30 (25–36)	30 (25–36)	35 (28–43)	<0.001*
Pulmonary congestion	49%	49%	11%	<0.001*
Peripheral oedema	55%	49%	24%	<0.001*
Systolic blood pressure (mmHg)	122 (110–140)	122 (110–140)	123 (110–140)	0.654
Diastolic blood pressure (mmHg)	75 (68–85)	75 (68–85)	75 (66–80)	0.011*
Heart rate (beat/min)	75 (65–88)	75 (65–88)	70 (61–80)	<0.001*
Beta-blocker	85%	85%	93%	<0.001*
ACE inhibitor or ARB	74%	74%	89%	<0.001*
Haemoglobin (g/dL)	13.4 (12.1-14.5)	13.4 (12.1-14.5)	13.3 (12.1-14.3)	0.030*
Urea (mmol/L)	9.4 (6.8-14.3)	9.4 (6.8-14.3)	10.3 (7.1-15.7)	<0.001*
eGFR* (ml/min/1.73m^2)	66 (49-82)	66 (49-82)	61 (46-79)	<0.001*
Sodium (mmol/L)	140 (137-142)	140 (137-142)	139 (137-142)	0.209
BNP (pg/mL)	202 (85-406)	202 (85-406)	134 (49-349)	0.001*
BNP 5-32	0.17 (0.00-0.49)	0.15 (0.00-0.48)	0.00 (0.00-0.00)	<0.001*
BNP 4-32	0.00 (0.00-0.31)	0.00 (0.00-0.30)	0.00 (0.00-0.00)	<0.001*
BNP 3-32	0.00 (0.00-0.34)	0.00 (0.00-0.34)	0.00 (0.00-0.00)	<0.001*
Endpoints				
1 year				
Death	19			
Death/HF	192			
2 years				
Death	118			
Death/HF	311			
3 years				
Death	142			
Death/HF	327			

Combined data is shown as median (interquartile range) for continuous variables and as a % for categorical. P values are quoted for Wilcoxon matched-pair signed-rank tests for continuous variable and McNemar test for categorical variables. BNP molecular forms are reported as MS ion signal intensity ratio against an internal reference standard (/ACTH) (5 fmol/µL). CKD-EPI formula was used to estimate eGFR.

ACE; angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BNP, B-type natriuretic peptide; BP, blood pressure; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; HF, heart failure; LV, left ventricular; NYHA, New York Heart Association

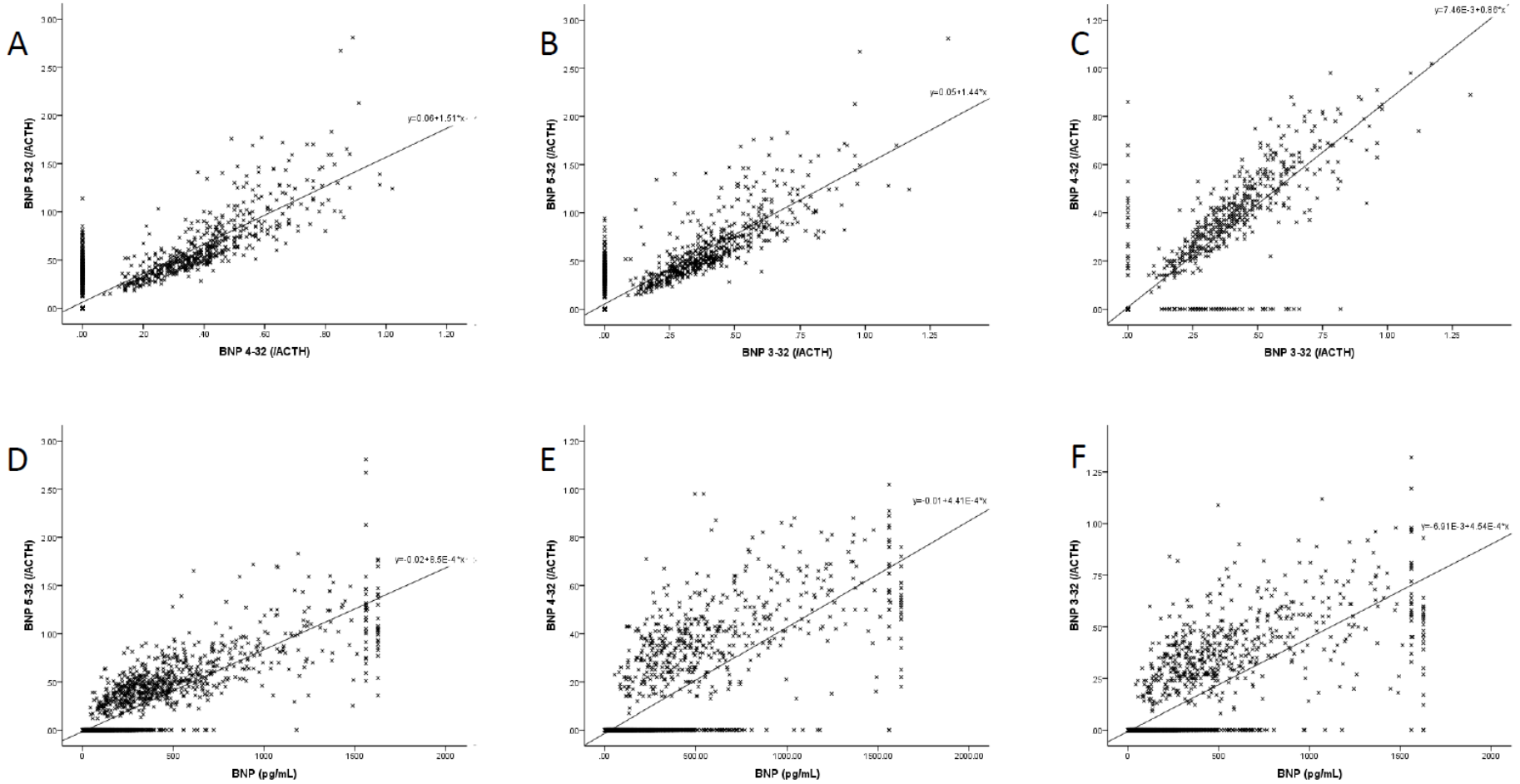


Figure 4.2 Correlation between BNP molecular forms (r_s = 0.838-0.901) and BNP (r_s = 0.674-0.787). A- BNP 5-32 and BNP 4-32, B- BNP 5-32 and BNP 3-32, C- BNP 4-32 and BNP 3-32, D- BNP and BNP 5-32, E- BNP and BNP 4-32, F- BNP and BNP 3-32

Table 4.2 BNP molecular forms and associated clinical factors for visit 1 and visit 2 data

Visit 1 (n=1127)	BNP 5-32		BNP 4-32		BNP 3-32		BNP	
	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value
Urea	0.116	<0.001	0.272	<0.001	0.271	<0.001	0.324	<0.001
eGFR	-0.159	<0.001	-0.343	<0.001	-0.329	<0.001	-0.376	<0.001
Heart Rate	0.169	<0.001	0.138	<0.001	0.149	<0.001	0.175	<0.001
Systolic BP	-0.136	<0.001	-0.142	<0.001	-0.125	<0.001	-0.132	<0.001
Age	0.101	0.001	0.049	0.010	0.053	0.073	0.170	<0.001
Blood Sodium	-0.088	0.006	-0.080	0.012	-0.064	0.043	-0.176	<0.001
Diastolic BP	-0.046	0.125	-0.047	0.115	-0.025	0.400	-0.082	0.006
BNP 5-32			0.829	<0.001	0.870	<0.001	0.823	<0.001
BNP 4-32					0.897	<0.001	0.699	<0.001
BNP 3-32							0.727	<0.001
Visit 2 (n=757)	**BNP 5-32**		**BNP 4-32**		**BNP 3-32**		**BNP**	
	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value
Urea	0.232	<0.001	0.225	<0.001	0.220	<0.001	0.324	<0.001
eGFR	-0.263	<0.001	-0.221	<0.001	-0.212	<0.001	-0.379	<0.001
Age	0.126	<0.001	0.097	0.007	0.091	0.012	0.328	<0.001
Systolic BP	-0.129	<0.001	-0.129	<0.001	-0.118	<0.001	-0.131	<0.001
Diastolic BP	-0.138	<0.001	-0.103	<0.001	-0.099	<0.001	-0.203	<0.001
Blood Sodium	-0.052	0.164	-0.060	0.110	-0.061	0.103	-0.050	0.179
Heart Rate	-0.034	0.354	-0.027	0.466	-0.015	0.672	-0.095	0.009
BNP 5-32			0.849	<0.001	0.840	<0.001	0.733	<0.001
BNP 4-32					0.904	<0.001	0.633	<0.001
BNP 3-32							0.624	<0.001

Table showing the associations and correlations between traditional clinical factors and BNP molecular forms for visit 1 and visit 2 data

BP, blood pressure; eGFR, estimated glomerular filtration rate; Spearman's rho, r_s

Table 4.3 Associations between BNP molecular forms and BNP combining V1 and V2 (n=1884), and also when adjusting for detection of molecular form

	BNP 5-32		BNP 4-32		BNP 3-32	
	All measured (r_s)	Detected only (r_s)	All measured (r_s)	Detected only (r_s)	All measured (r_s)	Detected only (r_s)
BNP 5-32			0.838	0.875	0.864	0.842
BNP 4-32	0.838	0.584			0.901	0.795
BNP 3-32	0.864	0.549	0.901	0.842		
BNP	0.787	0.747	0.674	0.614	0.674	0.583

Table showing the associations between BNP molecular forms and BNP in the full cohort (V1 and V2, n=1884) and also when selecting each BNP molecular form individually based on detection (i.e. >0)

Spearman's rho, r_s

4.3.3 BNP molecular forms and association with adverse outcomes

Cumulative incidence between BNP molecular forms and outcomes was investigated and Kaplan-Meier analyses performed across BNP molecular forms and BNP medians for 3 year composite of death and hospitalisation due to HF (death/HF). During this period, there were 327 events (29%). The results showed that elevated levels of BNP molecular forms and BNP were associated with more composite events, and an increase in cumulative incidence with increasing levels ($p<0.01$) (Figure 4.3).

Cox survival analysis was used to investigate the prognostic ability of BNP molecular forms for death and death/HF at 1 year, 2 years and 3 years adjusted using a multivariate model. The BIOSTAT-CHF compact and extended risk models were employed. The BIOSTAT-CHF compact risk model for mortality included age, haemoglobin, blood urea and use of beta-blocker at baseline. Log BNP was also used, but substituted for each of the molecular forms. BNP molecular forms were used as categorical variables (detection of the peak) rather than continuous (/ACTH). When adjusted for this model, BNP molecular forms were associated with mortality at 2 and 3 years (HR 1.64-2.19 $p\leq0.009$). BNP 5-32 was the more superior of the molecular forms and showed comparable qualities to BNP (HR 1.86-2.19, $p\leq0.002$ for BNP 5-32, HR 1.89-1.92, $p\leq0.004$ for BNP). The extended model for mortality included those variables described in the compact model and ischaemic aetiology, previous chronic obstructive pulmonary disease, diastolic blood pressure and sodium. BNP molecular forms remained independent predictors after adjustment for mortality at 2 and 3 years, comparable to BNP (HR 1.58-2.11 $p\leq0.019$ vs HR 1.81-1.86 $p\leq0.009$).

The compact risk model for death/HF included age, previous HF hospitalisation, peripheral oedema, systolic blood pressure, haemoglobin, sodium and use of beta-blocker at baseline. HDL was excluded from the model due to insufficient data. When adjusted for this model, BNP molecular forms were associated with mortality at 1, 2 and 3 years (HR 1.32-1.86 $p\leq0.031$). BNP 5-32 was the more superior of the molecular forms and showed comparable qualities to BNP (HR 1.48-1.71, $p\leq0.003$ for BNP 5-32, HR 1.62-1.71, $p\leq0.007$ for BNP). The extended risk model for mortality/HF included current smoking, previous chronic obstructive pulmonary disease and estimated glomerular

filtration rate (eGFR) in addition to the variables of the compact model. BNP molecular forms remained independent predictors after adjustment for mortality at 1, 2 and 3 years, comparable to BNP (HR 1.30-1.70 p≤0.046 vs HR 1.55-1.68 p≤0.017). BNP 4-32 was not a predictor at 3 years for death/HF (p=0.067).

Interestingly, BNP molecular forms showed improvements in HR for predictive abilities at short-term prognosis (1 year death/HF) (HR 1.27-1.71). BNP showed increased HR at long-term prognosis (3 years) (HR 1.55-1.71). Suggesting a role for BNP molecular forms for short-term prognosis. These results are detailed in Table 4.4.

BNP molecular forms showed added value to risk models for association with death at 2 and 3 years in both the compact and extended models, and for death/HF at 1, 2 and 3 years with the exception of BNP 4-32.

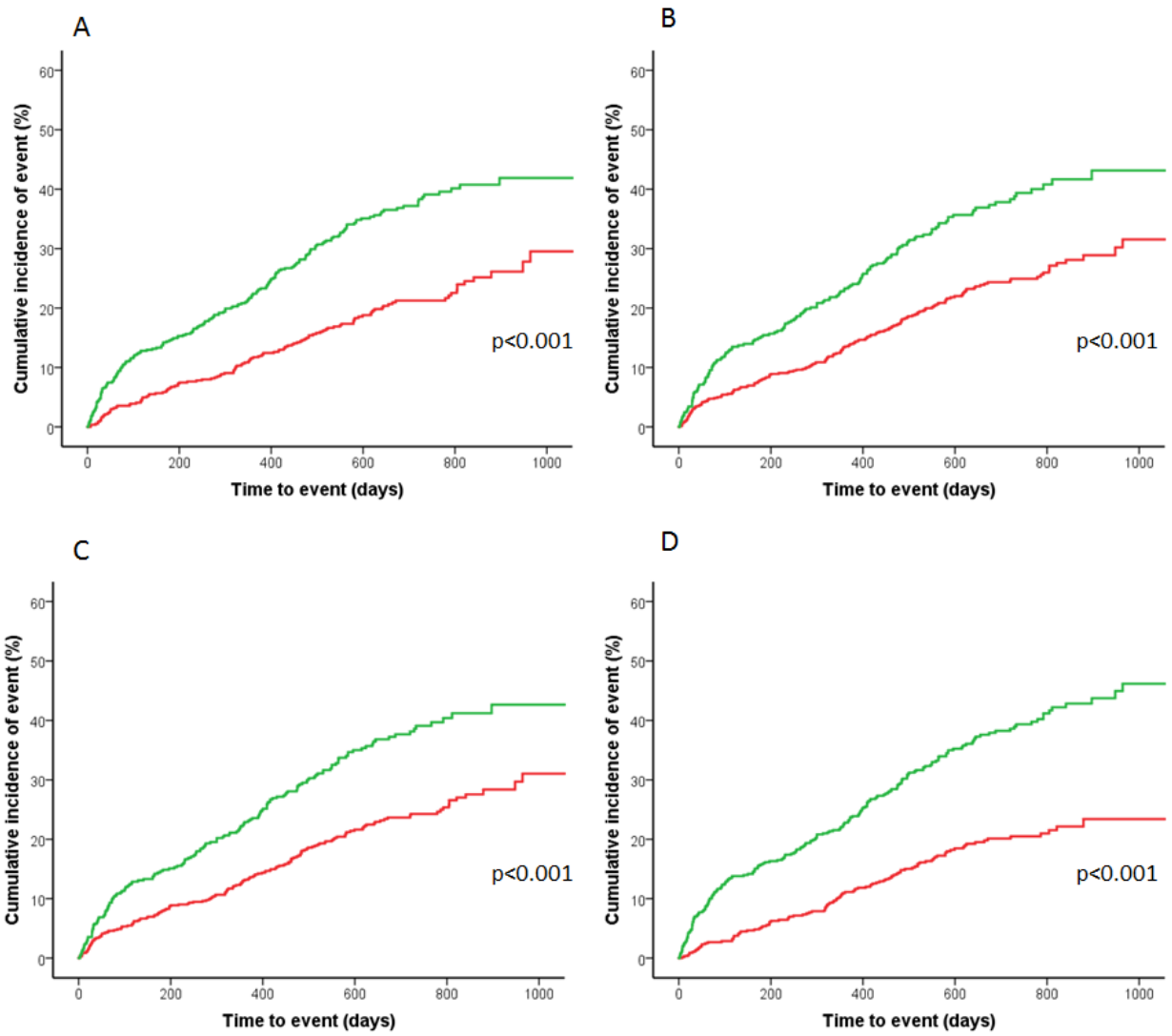


Figure 4.3 Kaplan-Meier curves showing **c**umulative incidence of events for 3 year composite events of mortality/HF for BNP molecular forms and BNP after stratification by medians.

A- BNP 5-32, **B**- BNP 4-32, **C**- BNP 3-32, **D**- BNP

Green line – above median; Red line – below median

Table 4.4 Independent prediction abilities of BNP molecular forms using multivariate Cox survival analyses for outcomes of death and death/HF at 1 year, 2 years and 3 years.

BNP 5-32	Compact Model			Extended Model		
	HR	95% CI	p Value	HR	95% CI	p Value
Death/HF 3yr	1.48	1.14-1.92	0.003	1.46	1.12-1.90	0.005
Death/HF 2yr	1.59	1.21-2.08	0.001	1.56	1.19-2.05	0.001
Death/HF 1yr	1.71	1.21-2.43	0.003	1.70	1.19-2.42	0.003
Death at 3yr	1.86	1.26-2.75	0.002	1.82	1.23-2.70	0.003
Death at 2yr	2.19	1.41-3.39	<0.001	2.11	1.36-3.27	0.001
Death at 1yr	1.13	0.40-3.24	0.818	1.01	0.34-2.99	0.988

BNP 4-32	Compact Model			Extended Model		
	HR	95% CI	p Value	HR	95% CI	p Value
Death/HF 3yr	1.32	1.03-1.70	0.031	1.27	0.98-1.64	0.067
Death/HF 2yr	1.36	1.05-1.76	0.019	1.31	1.01-1.90	0.045
Death/HF 1yr	1.50	1.08-2.08	0.016	1.44	1.03-2.01	0.033
Death at 3yr	1.64	1.14-2.37	0.008	1.58	1.09-2.28	0.015
Death at 2yr	1.71	1.14-2.55	0.009	1.62	1.08-2.41	0.019
Death at 1yr	1.02	0.36-2.92	0.965	1.02	0.35-2.95	0.974

BNP 3-32	Compact Model			Extended Model		
	HR	95% CI	p Value	HR	95% CI	p Value
Death/HF 3yr	1.34	1.05-1.73	0.021	1.30	1.01-1.68	0.046
Death/HF 2yr	1.39	1.07-1.79	0.013	1.34	1.03-1.74	0.031
Death/HF 1yr	1.57	1.13-2.18	0.007	1.52	1.09-2.13	0.014
Death at 3yr	1.67	1.16-2.41	0.006	1.65	1.14-2.38	0.008
Death at 2yr	1.80	1.20-2.68	0.004	1.74	1.16-2.61	0.007
Death at 1yr	1.11	0.40-3.08	0.849	1.19	0.42-3.39	0.750

BNP	Compact Model			Extended Model		
	HR	95% CI	p Value	HR	95% CI	p Value
Death/HF 3yr	1.71	1.30-2.26	<0.001	1.68	1.27-2.22	<0.001
Death/HF 2yr	1.71	1.29-2.26	<0.001	1.67	1.26-2.22	<0.001
Death/HF 1yr	1.62	1.14-2.32	0.007	1.55	1.08-2.21	0.017
Death at 3yr	1.92	1.28-2.88	0.002	1.86	1.24-2.79	0.003
Death at 2yr	1.89	1.22-2.93	0.004	1.81	1.16-2.81	0.009
Death at 1yr	0.70	0.26-1.87	0.471	0.71	0.27-1.87	0.492

Compact risk model for mortality adjusted for age, haemoglobin, blood urea and use of beta-blocker at baseline and the extended model for included ischaemic aetiology, previous chronic obstructive pulmonary disease, diastolic blood pressure and sodium.

The compact risk model for mortality/HF included age, previous HF hospitalisation, peripheral oedema, systolic blood pressure, haemoglobin, sodium and use of beta-blocker at baseline and the extended risk model for mortality/HF included current smoking, previous chronic obstructive pulmonary disease and estimated glomerular filtration rate (eGFR) in addition to the variables of the compact model

CI, confidence interval; BNP; B-type natriuretic peptide; HF, heart failure; HR, hazard ratio

4.3.4 Association of serial BNP 5-32 changes with adverse outcomes

As BNP 5-32 was the most superior molecular form due to its superior detection rate compared to BNP 4-32 and BNP 3-32, and better prognostic abilities, further comparative analyses against BNP were conducted using only BNP 5-32.

To analyse the associations of adverse events with serial changes in BNP 5-32 levels, BNP 5-32 data was split into four groups in relation to the ability to detect at visit 1 and visit 2, nine months after treatment. The groups were comprised of no detection in both visits (No/No n=327), no detect in visit 1 and detect in visit 2 (No/Yes n=47), detect in visit 1 and no detection in visit 2 (Yes/No, n=242), and detection in both visits (Yes/Yes, n=142). The results showed that patients with detection of BNP 5-32 in the second visit (No/Yes and Yes/Yes) showed an increased association with mortality at 2 years, compared to the reference No/No group [HR (95% confidence intervals) p-value; 2.36 (1.10-5.05) p=0.029 and 4.09 (2.49-6.74) p<0.001 for No/Yes and Yes/Yes respectively]. However, patients who showed BNP 5-32 detection at enrolment (visit 1) but no detection at follow-up (Yes/No) did not show an increased association with mortality at 2 years follow-up compared to the reference [1.16 (0.66-2.05) p=0.603] (Figure 4.4). Hence, increased association with mortality was observed in patients with detectable BNP 5-32 at follow-up, conversely better prognosis was observed in patients who did not have detectable BNP 5-32 at follow-up; regardless of detection at enrolment.

A similar trend was observed for BNP levels. Patients were split into four groups by median BNP levels for V1 (202 pg/mL) and V2 (134 pg/mL). The groups were comprised of below median in both visits (L/L n=299), below median in visit 1 and above median in visit 2 (L/H n=82), above median in visit 1 and below median in visit 2 (H/L, n=167), and above median in both visits (H/H, n=209). Analysis showed that patients with high BNP levels at follow-up (L/H and H/H) showed an increased association with mortality compared with the reference group (L/L) in a similar manner to No/Yes and Yes/Yes for BNP 5-32 [2.26 (1.15-4.43) p=0.018; 3.39 (1.94-5.92) p<0.001 for L/H and H/H respectively]. However, patients who showed elevated levels at enrolment but low levels at follow-up (H/L) did not show an increased association with mortality compared to the reference, in a similar manner to Yes/No for BNP 5-32 [1.10 (0.49-2.49) p=0.816]

(Figure 4.4). Both BNP 5-32 and BNP showed similar trends that increased association with mortality was governed by the follow-up such that those with BNP 5-32 detection or high BNP at visit 2 showed worse prognosis.

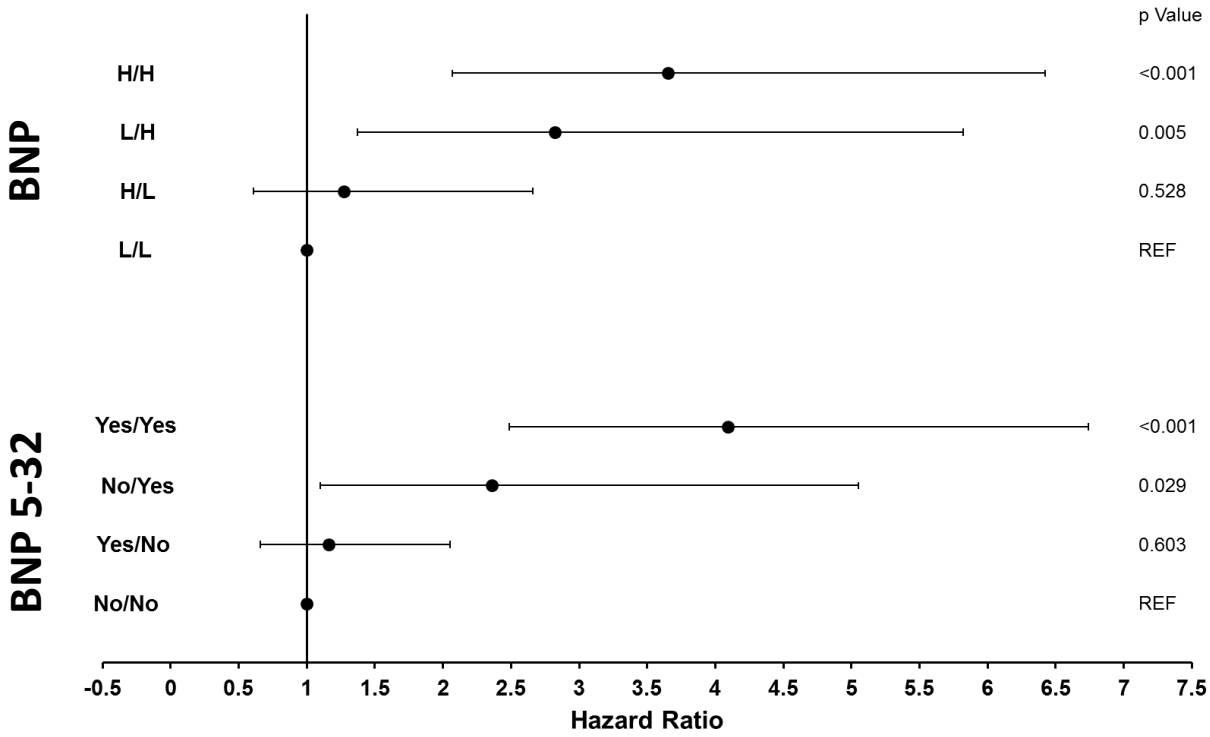


Figure 4.4 Forest plot showing the association with outcome for patients with BNP (top) and BNP 5-32 (bottom) measured at enrolment (visit 1) and 9 month follow-up (visit 2).

Patients were divided into four groups according to detection for BNP 5-32:no detection in both visits (No/No), no detect in visit 1 and detect in visit 2 (No/Yes), detect in visit 1 and no detection in visit 2 (Yes/No), and detection in both visits (Yes/Yes) and relative to the median BNP for each visit (202 pg/mL, visit 1; 134 pg/mL, visit 2); (L/L), below median in visit 1 and above median in visit 2 (L/H), above median in visit 1 and below median in visit 2 (H/L), and above median in both visits (H/H). Cox proportional hazards regression was used to compare the risk of mortality at 2 years after follow-up of patients using the L/L (BNP) and No/No (BNP 5-32) as the reference. Data presented as hazard ratio (HR) and 95% confidence interval (CI).

4.3.5 Effects of treatment monitoring

Changes in dosage titrations and the response of BNP 5-32 was investigated. Patients using ACEi, beta-blockers and both ACEi/beta-blocker, regardless of up- or down-titration, BNP 5-32 was increased by V2 ($p<0.001$). In contrast, significantly reduced levels of BNP were observed and showed responsiveness to guideline-based HF treatment when at least one of the ACEi/ARBs, beta-blocker or both ACEi/ARBs-beta-blocker were up-titrated ($p\geq359$) (Table 4.5). BNP 5-32 was not responsive to optimised guideline-based HF treatment in contrast to BNP levels.

Table 4.5 Response to guideline-based treatment for BNP 5-32 and BNP

	BNP 5-32 (n=757)			BNP (n=757)		
Dose up-titration	V1 (/ACTH)	V2 (/ACTH)	p Value	V1 (pg/mL)	V2 (pg/mL)	p Value
ACEi						
<50%	0.3 [0.0–0.5]	0.0 [0.0–0.3]	<0.001*	228 [100–467]	161 [69–420]	0.359
≥50%	0.0 [0.0–0.5]	0.0 [0.0–0.0]	<0.001*	169 [77–344]	114 [39–283]	0.001*
Beta-blocker						
<50%	0.2 [0.0-0.5]	0.0 [0.0-0.3]	<0.001*	183 [85–390]	142 [54–382]	0.389
≥50%	0.0 [0.0-0.5]	0.0 [0.0-0.0]	<0.001*	208 [88–413]	125 [43–291]	<0.001*
Both drugs						
Both <50%	0.2 [0.0–0.5]	0.0 [0.0-0.3]	<0.001*	200 [85–408]	141 [56–382]	0.362
Both ≥50%	0.0 [0.0-0.5]	0.0 [0.0-0.0]	<0.001*	206 [86–391]	121 [37–251]	<0.001*

ACEi, angiotensin-converting enzyme inhibitor; ACTH, adrenocorticotropic hormone; BNP, B-type natriuretic peptide; V1, visit 1 (enrolment); V2, visit 2 (9 month follow-up)

<50% less than 50% of optimal recommended dosage

≥50% of optimal recommended dosage

4.3.6 Decision tree analysis

Decision tree analysis was performed to assess the utility of BNP 5-32 at V1 and V2 as a risk stratification biomarker for death at 2 years following V2. Results showed that a primary BNP 5-32 value of 0.340 (event risk 9.2% below 0.340, event risk 18.2% above 0.340) identified a total of five further groups of risk. Firstly, those with a value below 0.340 identified two groups of risk. At follow-up, these groups were stratified by a BNP 5-32 value of 0.000 (i.e even based on continuous data this expresses no detectable BNP 5-32 at V2). Those below this value showed a group event risk of 7.5% (Group A) whereas those above this threshold showed a group event risk of 19.7% (Group B). Furthermore, those with a BNP 5-32 V1 value above 0.340 were split into three further groups at follow-up, stratified by V2 BNP 5-32 value below 0.000 (Group C), between 0.000-0.560 (Group D), and above 0.560 (Group E). Interestingly, Group C had an event risk of 10.5%, Group D 21.2%, and Group E 40% (Figure 4.5). This showed that event risk and worse prognosis was governed by BNP 5-32 values at V2, irrespective of initial V1 values with Group's A and C with <0.000 (risk≤10.5%) BNP 5-32 value and Group's B, D, and E >0.000 (risk≥19.7%) showing increasing event risk and confirming associations reported by the previous Cox proportional hazards regression (Figure 4.4). Kaplan-Meier survival analysis confirmed that Group E had an increased number of cumulative events compared to the other groups (p≤0.001) (Figure 4.5, inset).

Decision tree analysis for BNP showed similar results. At enrolment (V1), BNP levels of 142.5 pg/mL showed stratification of two groups; below threshold (event risk 7.9%) and above threshold (event risk 16.1%). Below the threshold group was split into two further groups at follow-up with BNP levels of 64.2 pg/mL (Group's A and B), and above the V1 threshold identified three groups Group C (BNP≤93.3 pg/mL), Group D (93.4-702.9 pg/mL), and Group E (BNP>702.9 pg/mL). Similar to BNP 5-32, this showed that event risk and worse prognosis irrespective of V1 measurements was governed by BNP levels at V2 with Group's A and C with ≤93.3 pg/mL (risk≤5.5%) BNP value and Group's D, and E >93.3 pg/mL (risk>16.0%) showing increasing event risk and confirming associations reported by the previous Cox proportional hazards regression (Figure 4.4). Kaplan-Meier survival analysis confirmed that Group E had an increased number of cumulative events compared to the other groups (p≤0.001) (Figure 4.6, inset).

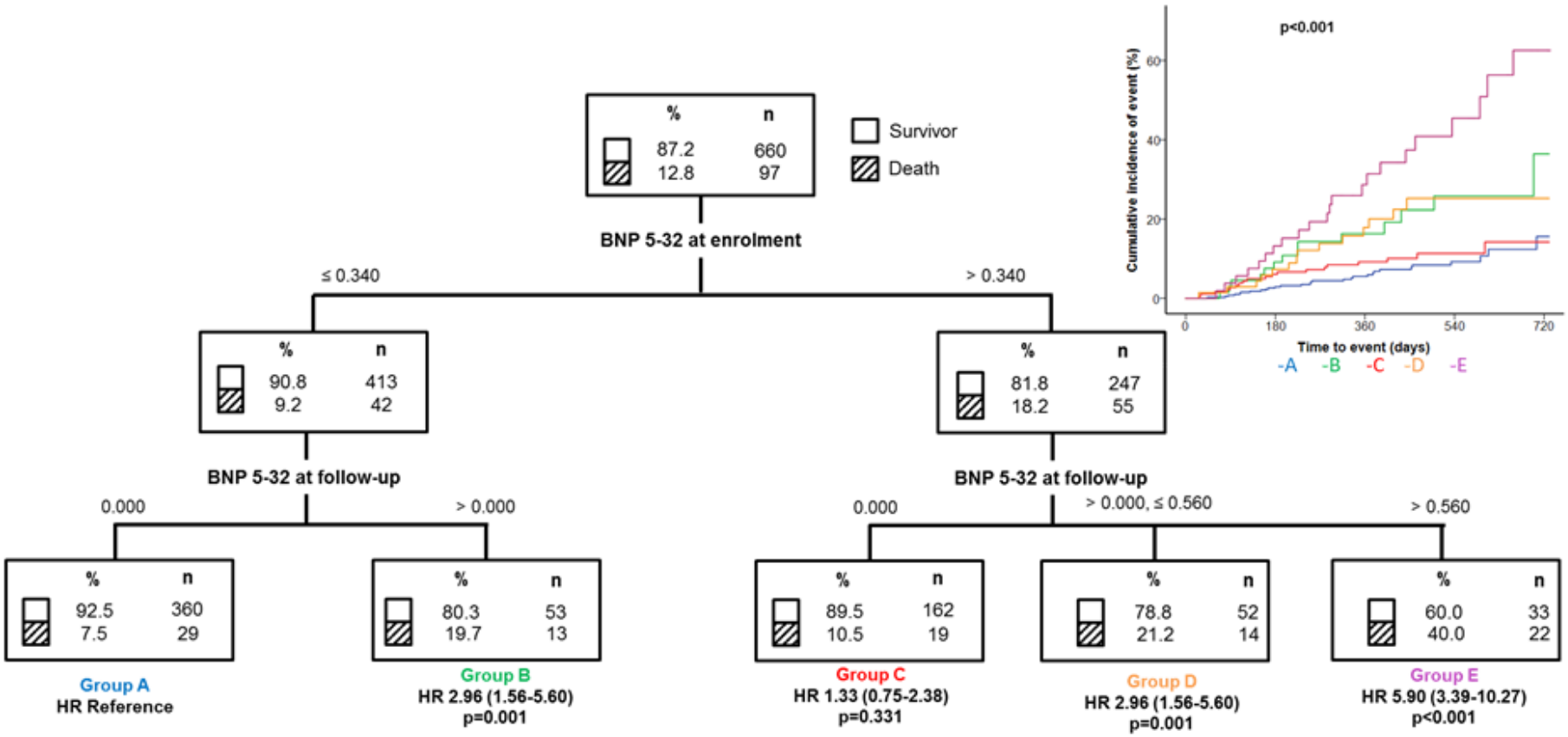


Figure 4.5 Decision tree showing risk stratification for death at 2 years using combined measurements at baseline (enrolment, V1) and follow-up (V2) for BNP 5-32, and cumulative event incidence of risk groups (inset). Data presented as hazard ratio (HR) and 95% confidence interval

BNP, B-type natriuretic peptide; HR, hazard ratio

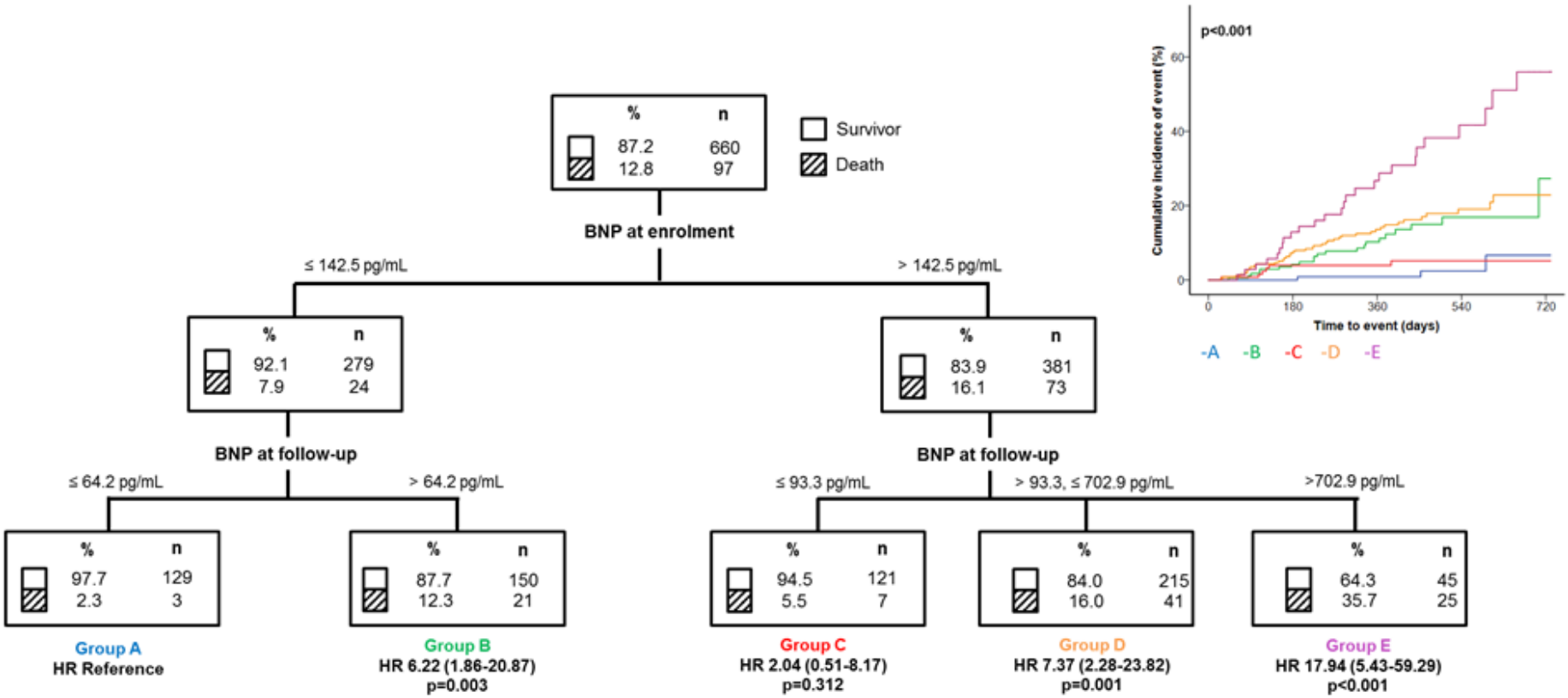


Figure 4.6 Decision tree showing risk stratification for death at 2 years using combined measurements at baseline (enrolment, V1) and follow-up (V2) for BNP, and cumulative event incidence of risk groups (inset). Data presented as hazard ratio (HR) and 95% confidence interval

BNP, B-type natriuretic peptide; HR, hazard ratio

4.4 Discussion

This study investigated the role of BNP molecular forms in the large multi-centre BIOSTAT-CHF cohort, designed to characterise biological pathways related to response to therapy for HF (Voors et al., 2016). This cohort, in contrast to the acute HF and MI cohorts, enrolled patients with worsening or new-onset of patients with HF and all patients received targeted treatment (ACE/beta-blocker) to monitor changes at follow-up. This study reports that BNP molecular forms were associated with adverse events of death and a composite of death and hospitalisation due to HF. When stratified by the median at enrolment, BNP molecular forms showed increased risk of composite event and incidence with elevated circulating levels.

BNP molecular forms were all independently able to predict death/HF at 1, 2 and 3 years when adjusted using the BIOSTAT-CHF compact model, and further BNP 5-32 and BNP 3-32 were able to retain their predictive abilities when adjusted by the extended model (Table 4.4). For death, BNP molecular forms were independent predictors at 2 and 3 years. Comparable qualities were shown by clinical BNP measurements. Interestingly, for death/HF the prognostic abilities for BNP molecular forms were improved at short-term (1 year) compared with long-term prognosis. In contrast, the opposite of this was observed with BNP where its independent prognostic abilities were improved over time. This suggests that BNP molecular forms were able to add to the BIOSTAT-CHF risk models and also suggests BNP molecular forms as a role in short-term prognosis of HF.

BNP molecular forms showed strong associations between themselves and also with BNP (Figure 4.2). It was also observed that while BNP molecular forms were associated with heart rate at enrolment, at follow-up this association was not observed. In contrast, diastolic blood pressure was associated with BNP molecular forms at enrolment, but not a follow-up (Table 4.2).

To monitor response to treatment, patients underwent treatment with furosemide ≥40mg/day or equivalent and received ≤50% doses of ACEi/ARBs and beta-blockers at

time of enrolment which were then up-titrated in the next three months, and recorded to allow for analysis.

As BNP 5-32 was the most discriminatory of molecular forms and showed superior detection rate, it was taken forward in further analysis. Follow-up data showed that by assessing the changes from enrolment, the observation for BNP and BNP 5-32 were similar. Both biomarkers showed that increased association with death was observed in patients with detectable BNP 5-32/BNP at follow-up regardless of detection at enrolment. A better prognosis was observed in patient's null of BNP 5-32 (Figure 4.4). The results for BNP 5-32 are in support of previous studies for natriuretic peptide levels where serial measurements was associated with improved outcome (Latini et al., 2006).

The effects of treatment monitoring showed that BNP 5-32 was not responsive to guideline-based HF treatment showing a change between enrolment and follow-up regardless of drug titration. In contrast BNP was responsive to treatment and showed reduced levels when at least one of ACEi/ARBS, beta-blocker or both were up-titrated consistent with previous findings (Troughton et al., 2014) showing the ability of BNP levels to decrease over time in response to combination therapy (Rosenberg et al., 2008) (Table 4.5).

In addition to this BNP 5-32 was able to identify patients at high risk for death at 2 years following visit 2 (Figure 4.5). It was successfully able to show that elevated BNP 5-32 levels at visit 2 were associated with increasing risk, irrespective of visit 1 values, comparable to BNP. This could suggest that BNP molecular forms perhaps lack the ability of prognosis in chronic HF, when BNP levels are stable and generally lower. This suggests the role of BNP 5-32 in risk stratification at follow-up and its utility in identifying the highest risk group showing more than two-fold increased risk of cumulative events than other groups. The results from this study also suggest the importance and utility of serial measurements of BNP 5-32 to monitor risk. This supports previous reports of serial measurements and risk stratification in chronic HF with NT-proBNP (Masson et al., 2008) and BNP (Zile et al., 2016).

It was interesting to find that current guideline-based treatment in HF impacted BNP levels, but not BNP 5-32. BNP levels were found to decrease in response to up-titration

(≥50%). This could be part due to the low detection rate certainly in the follow-up patients. As current recommended therapy for HF used in the BIOSTAT-CHF cohort did not affect BNP 5-32, the levels of BNP 5-32 could show responsive monitoring by alternative pathway interactions of other drugs, such as Entresto™. Further analysis is warranted to understand the impact of serial BNP molecular forms.

4.5 Conclusion

In conclusion, this study reports that BNP molecular forms were able to improve risk stratification of HF in the BIOSTAT-CHF cohort with elevated levels associated with adverse outcomes. Circulating plasma levels of BNP 5-32, BNP 4-32 and BNP 3-32 were associated with poor prognosis (death and death/HF) at 1, 2 and 3 years. Of the molecular forms, BNP 5-32 was the more superior and showed comparable results to BNP. Certainly at short-term prognosis, BNP 5-32 was more superior to BNP in predicting death/HF. Serial measurements of BNP 5-32 showed that patients with elevated levels at follow-up were associated with worse outcomes, irrespective of initial levels. BNP 5-32 was not responsive to guideline-based therapy in contrast to BNP, requiring further research for therapeutic monitoring. This study extends upon previous research identifying the importance of serial measurements of NPs for improved prognostic qualities in HF risk stratification, and suggests a role for BNP molecular forms in short-term prognosis.

CHAPTER FIVE

ACUTE MYOCARDIAL INFARCTION STUDY

This chapter has been published elsewhere

Israr MZ, Heaney LM, Ng LL*, Suzuki T*. 2018. B-type natriuretic peptide molecular forms for risk stratification and prediction of outcome after acute myocardial infarction. American Heart Journal, 200:37-43.

5. ACUTE MYOCARDIAL INFARCTION STUDY

5.1 Introduction

5.1.1 Introduction and Rationale

BNP and NT-proBNP have been shown to be successful markers of risk stratification and outcomes in patients following acute myocardial infarction (MI). For example, previous investigations have shown elevated levels of BNP or NT-proBNP are associated with in-hospital (Scirica et al., 2013) and short-term (30-day) mortality (Mega et al., 2004), as well as mortality at extended follow-up periods of 4 years or more (Omland et al., 1996, Richards et al., 2003, Omland et al., 2002). From a pathophysiological standpoint, measured circulating levels of BNP have also shown relationships with infarct size (Mayr et al., 2011), systolic dysfunction (Gunes et al., 2008), development towards heart failure (Darbar et al., 1996) and cardiac death (Fazlinezhad et al., 2011). Although synonymous with use in diagnostic and prognostic assays for heart failure (HF), BNP has been able to provide complementary clinical information beyond traditional MI biomarkers such as troponin and copeptin (Omland et al., 1996).

From the previous study it was shown that BNP molecular forms are detectable using mass spectrometry methods and show utility as a prognostic marker in acute HF. There is no data investigating risk stratification in acute MI using BNP molecular forms.

5.1.2 Aims and Hypothesis

- Characterise circulating BNP molecular forms in acute MI patients and investigate their prognostic ability to assist in risk stratification.
- Analyse the usefulness as risk prediction markers with an established measurement of circulating BNP (NT-proBNP) and test in combination with the established GRACE (Global Registry of Acute Coronary Events) risk prediction tool for MI (Eagle et al., 2004).

BNP molecular forms are hypothesised to display comparable prognostic applications and show risk stratification utility as established measurements of NT-proBNP. BNP 5-32 is hypothesised to be the dominating molecular form of BNP to show the more similar characteristics as NT-proBNP.

5.2 Methods

5.2.1 Study population

This cohort has been previously used and therefore the sample collection and study population criterion had been arranged prior.

Patients with acute MI were admitted to the University Hospitals of Leicester between August 2004 and April 2007 and enrolled. Each patient consented to have blood samples taken and outcomes surveyed. This study was approved by the local ethics committee and adhered to the Declaration of Helsinki.

Diagnosis of acute MI was made on the basis that all patients had a cTnI concentration above the 99th percentile, with at least one of the following: chest pain lasting >20 min or diagnostic serial electrocardiographic changes consisting of new pathological Q-waves or ST-segment and T-wave changes (Thygesen et al., 2007), excluding patients with malignancy, renal replacement therapy, or previous surgery within 1 month. Analysis of renal function, eGFR, was calculated from the simplified Modification of Diet in Renal Disease formula (Smilde et al., 2006). All patients received standard medical treatment and revascularization at the discretion of the attending physician.

For prognostic investigations, primary endpoints were composites of all-cause mortality or reinfarction (death/MI), all-cause mortality or rehospitalisation due to HF (death/HF), as well as death/MI and death/HF combined (MACE). Outcomes were measured in all patients for short-term (6 months) and long-term (1 year and 2 years) risk prediction.

The GRACE score, for outcomes at 6 months, was tested with the addition of BNP molecular forms for the end point of death/MI. End points were obtained by reviewing the local hospital databases and the Office of National Statistics Registry, and by telephone calls to patients, and those data were verified by reviewing medical records. One hundred percent follow-up was achieved.

This cohort was initially designed for the risk stratification of MI using cardiac peptides. The study presented in this thesis lies within the remit of the initial intentions of the study, looking at risk stratification in acute MI. First of all, because BNP/NT-proBNP is a

cardiac-specific peptide biomarker, and has shown to be successful in the risk stratification of MI, this study was designed to compare BNP molecular forms against BNP/NT-proBNP in MI. This allows direct comparisons between BNP and BNP molecular forms to be made. In comparison to the acute HF study, this cohort initially had NT-proBNP concentrations available for comparison with BNP molecular forms, however due to the availability of the commercially available BNP kits, BNP concentrations were also measured in a subset of samples for direct BNP:BNP molecular form comparisons. Lastly, this study had a large sample number (n=1078) allowing to better determine the impact of the study (i.e confidence intervals and margins of error).

The age of the samples was the first concern for this study considering they were over 10 years old, and their NT-proBNP concentrations were not considerably elevated as compared to the acute HF study. This was evident with a considerable decrease in % detection. From the three cohorts used in this thesis, this was the oldest cohort. Secondly, although studies have shown BNP to be correlated to MI, it is not the recommended specific marker therefore the specificity of BNP molecular forms for MI were lacking in this study certainly compared to the previous cohorts. The recommended marker for MI is troponin. Although samples were collected on admission, the time (hours) of sample collection is unknown.

5.2.2 Sample collection

Samples were collected at time of discharge in pre-chilled tubes containing EDTA and aprotinin, and centrifuged at 1500g for 20 min at 4°C. Plasma was aliquoted and stored at -80°C until analysis. For analysis, samples were thawed at 37°C, prepared, and analysed immediately.

5.2.3 BNP molecular form measurements

BNP molecular forms were measured and the results were compared to NT-proBNP values previously measured in all patients for other studies using a sandwich immunoassay (Omland et al., 2002). Inter-assay relative standard deviations for daily control experiments across the course of the study for synthetic BNP 5-32, BNP 4-32

and BNP 3-32 were 6.2%, 9.7% and 13.7%, respectively. RSD was controlled within an average of 10% for the molecular forms, as shown in Figure 5.1 the average RSD = 9.9%.

Samples where BNP molecular forms were not detected, their result was recorded as equal to the average value of the baseline noise:ACTH ratio (a value of 0.03). For clarification, the baseline noise intensity in comparison to ACTH and BNP 5–32 at a value of 0.210 can be seen in Figure 5.2.

Patient samples that showed detection of BNP 5-32 (n=617) were analysed for clinical BNP concentrations using a point-of-care device (RapidPIA™, Sekisui Medical Co.).

5.2.4 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (V24, IBM Corp., Armonk, New York, USA). Correlations between BNP molecular forms and with other clinical variables were performed using Spearman's correlations. Multivariate and univariate models were made to evaluate independent predictors of death/MI and death/HF and MACE at 6 months, 1 year and 2 years using Cox proportional hazard regressions analyses. Values for molecular forms of BNP and NT-proBNP were log transformed. Continuous reclassification analyses (Pencina et al., 2011) were used to assess the utility of adding molecular BNP forms and NT-proBNP to the GRACE score for risk assessment at 6 months. Decision tree analysis was performed using the χ^2 automatic interaction detection (CHAID). A p-value <0.05 was deemed statistically significant.

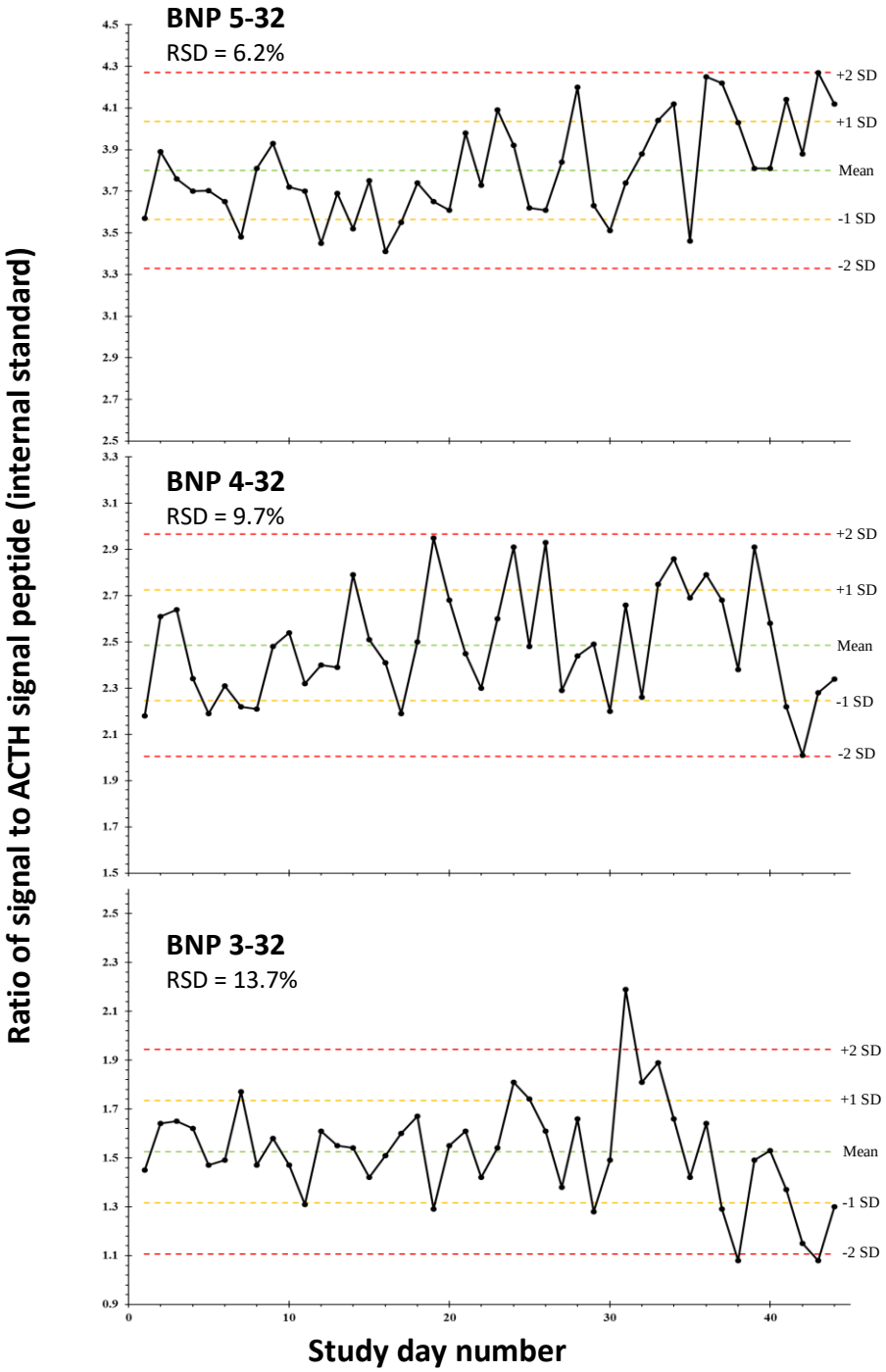


Figure 5.1 Relative Standard Deviation for synthetic BNP 5-32, BNP 4-32, and BNP 3-32 used as the positive control throughout the course of the study. Average RSD = 9.9% was less than the 10% limit and within three SD.

ACTH, Adrenocorticotrophic hormone; BNP, B-type natriuretic peptide; RSD, Relative standard deviation

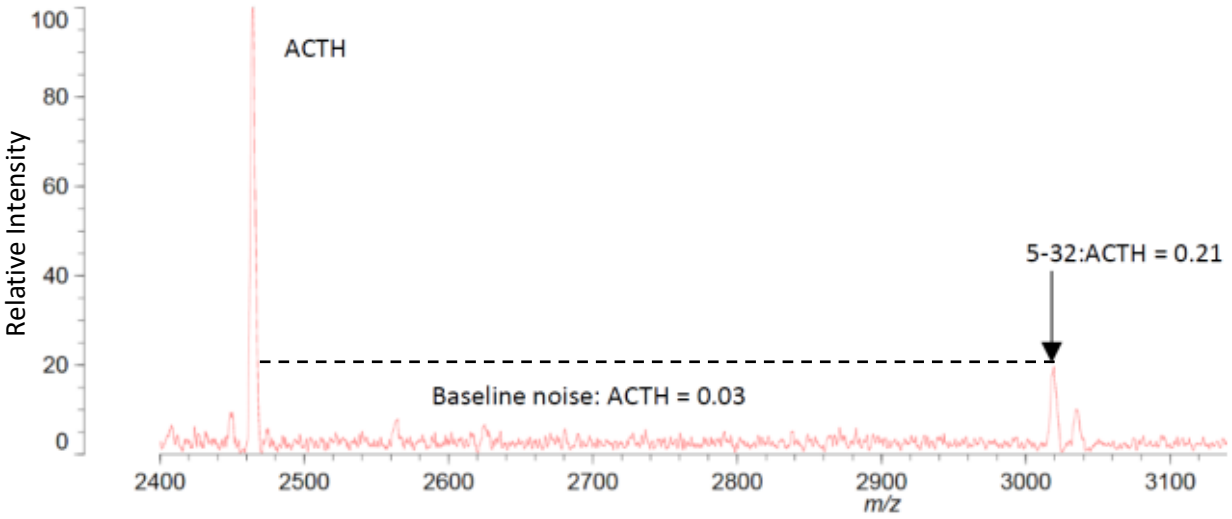


Figure 5.2 Example spectrum showing the ACTH signal, baseline noise and BNP 5-32 ratios.

ACTH, Adrenocorticotrophic hormone; BNP, B-type natriuretic peptide, m/z, mass-to-charge ratio

5.3 Results

5.3.1 Cohort characteristics and demographics

Plasma samples from 1,078 patients admitted to hospital with acute MI were analyzed for the presence of BNP molecular forms. BNP molecular forms were detected in a total of 617 (57.2%) of samples. A total of 292 events were recorded during the 2 year follow up period, with 232 deaths due to MI and 200 deaths from HF.

Analysis of trends in this cohort showed that the majority of the cohort were male (72%) and a median age of 67 years. The median NT-proBNP was extensively elevated at 813 pmol/L (6829 pg/mL, cut off for HF = 400 pg/mL). A breakdown of the measured endpoints, along with the clinical demographics for the patient cohort can be found in Table 5.1.

5.3.2 BNP molecular forms and associated clinical measurements

Univariate analysis showed clinical variables that correlated to one or more of the BNP molecular forms to be blood urea, eGFR, age, sex, cTnI, heart rate, blood glucose, blood sodium and systolic blood pressure, as detailed in Table 5.2. BNP molecular forms were strongly correlated to each other (r_s = 0.798-0.935, p<0.001) and modestly correlated to NT-proBNP (r_s = 0.591-0.640, p<0.001). The levels of correlation can be observed in Figure 5.3. The strongest level of correlation between the molecular forms was observed between BNP 4-32 and BNP 3-32 (r_s = 0.935) whereas BNP 5-32 showed the strongest level of correlation with NT-proBNP (r_s = 0.640). BNP molecular forms were correlated with markers of renal dysfunction including blood urea (r_s = 0.267-0.272, p<0.001) and eGFR (r_s = -0.323- -0.343, p<0.001) showing very similar comparisons with NT-proBNP; for blood urea (r_s = 0.324, p<0.001) and eGFR (r_s = -0.376, p<0.001). Only slight positive correlations were observed between the molecular forms and cTnI (r_s = 0.111-0.183, p<0.001), similar to the correlations between NT-proBNP and cTnI (r_s = 0.196, p<0.001).

A linear regression model was performed to investigate the independent predictors of BNP molecular forms (log-transformed) and NT-proBNP. Independent predictors for each of the BNP molecular forms can be found in Table 5.3. The results showed that

age, Killip >1, troponin and SBP were all independently predictive of all BNP molecular forms and NT-proBNP ($p \leq 0.048$), and BNP 5-32 and by eGFR levels ($p=0.032$) thus illustrating that renal dysfunction is indicated in the presence of BNP 5-32. Interestingly, whilst statins were predicative of the BNP molecular forms ($p \leq 0.001$), aspirin was independently predicative for NT-proBNP ($p=0.040$). As a clinically used marker of MI, troponin was a strong predictor for BNP molecular forms and NT-proBNP ($p<0.001$) alongside Killip >1 ($p<0.001$). STEMI was a strong predictor for NT-proBNP ($p<0.001$) and was also able to independently predict for BNP 5-32 ($p=0.048$).

Table 5.1 Patient demographics for acute MI patients at time of admission to hospital

Age (yrs)	67 (57-77)
Male	72%
Systolic BP (mmHg)	136 (120-151)
Diastolic BP (mmHg)	77 (68-88)
Heart rate (beats/min)	75 (63-95)
Past history MI/angina	33%
Past history hypertension	52%
Past history diabetes	23%
Past history HF	4%
Aspirin on discharge	84%
β-blockers on discharge	81%
ACE/ARB on discharge	84%
Statins on discharge	89%
Killip score >1	41%
ST elevation MI (%)	47%
Revascularization (%)	26%
Glucose (mmol/L)	7.5 (6.3-9.9)
Troponin (cTnI) (ng/mL)	3.6 (1.0-12.1)
Urea (mmol/L)	17 (14-22)
eGFR (mL/min/1.73m^2)	66 (53-78)
Na^+ (mmol/L)	138 (136-140)
K^+ (mmol/L)	4.2 (4.0-4.6)
NT-proBNP (pmol/L)	813 (260-2199)
GRACE score	120 (96-143)
BNP 5-32	0.2 (0.03-0.4)
BNP 4-32	0.04 (0.03-0.2)
BNP 3-32	0.04 (0.03-0.2)
Endpoints	
6 months	
Death/MI	161
Death/HF	146
MACE	209
1 year	
Death/MI	203
Death/HF	179
MACE	260
2 years	
Death/MI	232
Death/HF	200
MACE	292

Combined data is shown as median (interquartile range) for continuous variables and as a % for categorical data. BNP molecular forms are reported as MS ion signal intensity ratio against an internal reference standard (/ACTH) (5 fmol/µL)

BNP, B-type natriuretic peptide; BP, blood pressure; eGFR, estimated glomerular filtration rate; GRACE, Global Registry of Acute Coronary Events; HF, heart failure; MACE, major adverse cardiac event; MI, myocardial infarction, NT-proBNP, N-terminal pro B-type natriuretic peptide; NYHA, New York Heart Association

(Thygesen et al., 2007)

Table 5.2 BNP molecular forms and associated clinical factors

	BNP 5-32		BNP 4-32		BNP 3-32		NT-proBNP	
	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value
Urea	0.267	<0.001	0.272	<0.001	0.271	<0.001	0.324	<0.001
eGFR	-0.323	<0.001	-0.343	<0.001	-0.329	<0.001	-0.376	<0.001
Age	0.382	<0.001	0.396	<0.001	0.386	<0.001	0.450	<0.001
Sex	-0.165	<0.001	-0.189	<0.001	-0.176	<0.001	-0.162	<0.001
Troponin (cTnI)	0.183	<0.001	0.116	<0.001	0.111	<0.001	0.196	<0.001
Heart Rate	0.186	<0.001	0.213	<0.001	0.197	<0.001	0.144	<0.001
Blood Glucose	0.195	<0.001	0.157	<0.001	0.149	<0.001	0.222	<0.001
Blood Sodium	-0.088	0.006	-0.080	0.012	-0.064	0.043	-0.176	<0.001
Systolic BP	-0.074	0.020	-0.055	0.086	-0.057	0.073	-0.094	0.004
BNP 5-32			0.803	<0.001	0.798	<0.001	0.640	<0.001
BNP 4-32					0.935	<0.001	0.593	<0.001
BNP 3-32							0.591	<0.001

Table showing the associations and correlations between traditional clinical factors and BNP molecular forms. BNP molecular forms showed a strong positive correlation between themselves, whilst only a modest positive correlation with NT-proBNP. Compared to renal markers urea and eGFR, the BNP molecular forms showed similar correlations compared to NT-proBNP.

BNP, B-type natriuretic peptide; BP, blood pressure; eGFR, estimated glomerular filtration rate; NT-proBNP, N-terminal proBNP; r_s, Spearman's rho

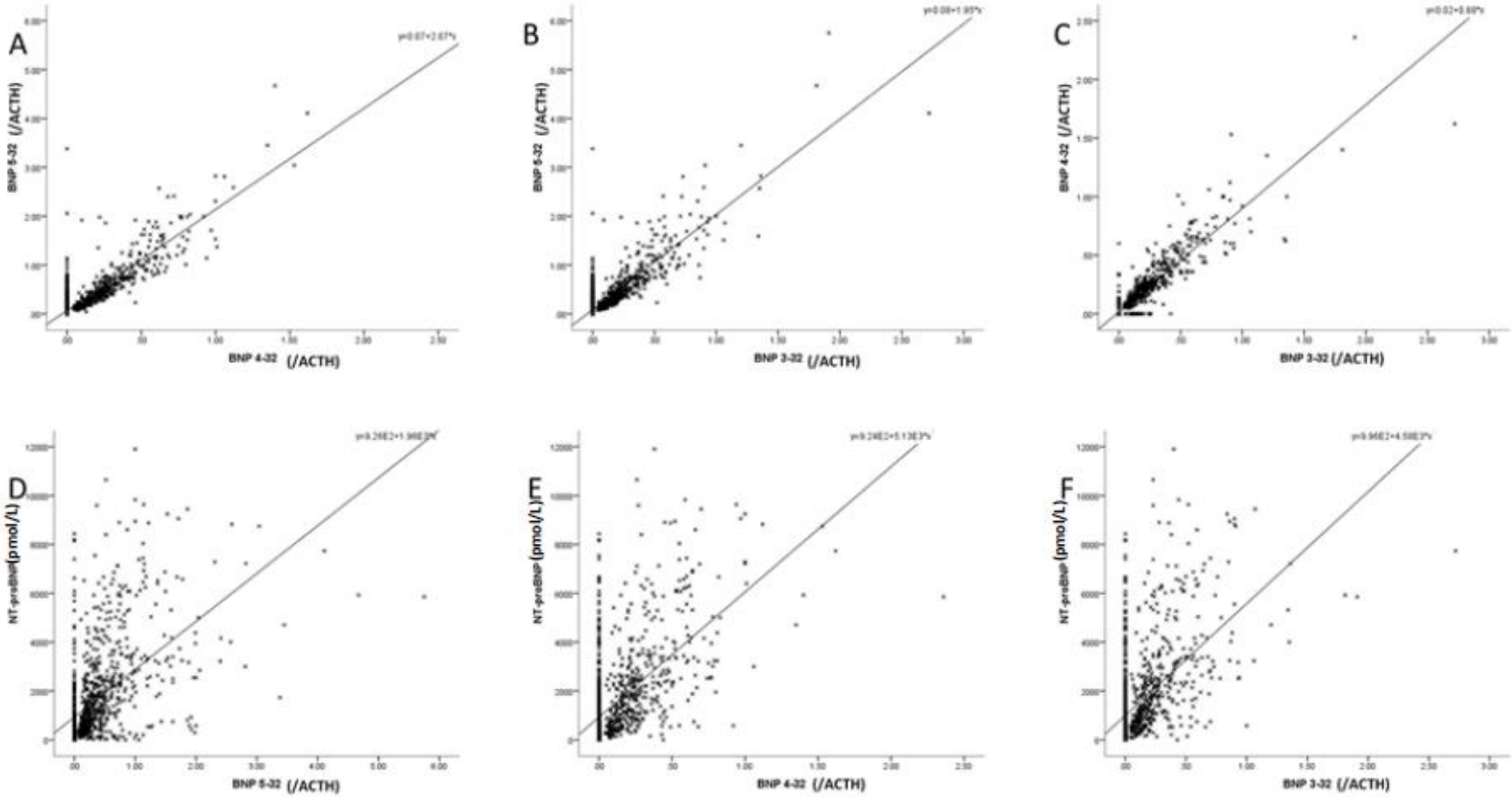


Figure 5.3 Correlation between BNP molecular forms (r_s = 0.798-0.935) and NT-proBNP (r_s = 0.591-0.640). A- BNP 5-32 and BNP 4-32, B- BNP 5-32 and BNP 3-32, C- BNP 4-32 and BNP 3-32, D- NT-proBNP and BNP 5-32, E- NT-proBNP and BNP 4-32, F- NT-proBNP and BNP 3-32

Table 5.3 Linear regression model for independent predictors of BNP molecular forms and NT-proBNP

BNP 5-32			**BNP 4-32**			**BNP 3-32**			**NT-proBNP**		
Variable	**Std β**	**p Value**	**Variable**	**Std β**	**p Value**	**Variable**	**Std β**	**p Value**	**Variable**	**Std β**	**p Value**
Age	0.274	<0.001	Age	0.222	<0.001	Age	0.213	<0.001	Age	0.380	<0.001
Killip >1	0.134	<0.001	Killip >1	0.151	<0.001	Killip >1	0.163	<0.001	Killip >1	0.145	<0.001
Troponin	0.175	<0.001	Troponin	0.143	<0.001	Troponin	0.135	<0.001	STEMI	0.138	<0.001
Statin	-0.117	0.001	Statin	-0.117	0.001	Statin	-0.141	0.001	Troponin	0.160	<0.001
eGFR	-0.089	0.032	Heart Rate	0.100	0.004	SBP	-0.072	0.039	Increased BP	0.095	0.006
Heart Rate	0.070	0.042	SBP	-0.075	0.029				Aspirin	-0.069	0.040
SBP	-0.068	0.048	Sex	-0.072	0.041				Heart Rate	0.070	0.042
STEMI	0.077	0.048							SBP	-0.091	0.048

The table shows that age, Killip >1, troponin and SBP were independent predictors for all BNP molecular forms and NT-proBNP. Interestingly, whilst statins on discharge were independent predictors of BNP molecular forms, aspirin was the independent predictor for NT-proBNP.

BP, blood pressure; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; STEMI, ST-elevation myocardial infarction; Std β, standardised beta

5.3.3 BNP molecular forms as predictors of death/MI

Cox survival analysis was conducted to investigate the prognostic ability of BNP molecular forms for death/MI at 6 months, 1 year and 2 years, using a multivariable model adjusted for traditional cardiovascular disease risk factors. The risk factors included in the model were age, sex, systolic BP, Killip score, STEMI class, revascularization, medication at discharge (aspirin, β-blockers, ACE/ARB, statins), renal markers (eGFR and urea), cTnI levels and past histories of MI/angina, diabetes and hypertension. Each marker was added to the base to predict outcome and analyse their independent abilities. Results showed that NT-proBNP was a univariate predictor of death/MI at 6 months, 1 year and at 2 years ($p<0.001$), and retained independent prediction at these end points ($p\leq0.020$) when added to the base model. Similarly, BNP molecular forms were univariate predictors of death/MI at 6 months, 1 year and at 2 years ($p<0.001$). When BNP molecular forms were added to the base model, they retained independent prediction at 6 months ($p\leq0.026$), 1 year ($p\leq0.018$) and at 2 years ($p\leq0.008$) showing comparable abilities to NT-proBNP. BNP 5-32 expressed the most superior prognostic qualities of the molecular forms (Table 5.4). Other independent predictors of outcome were age, urea and revascularisation.

ROC curves were comparable between molecular forms and NT-proBNP for death/MI at 6 months (AUC 0.63 to 0.69), 1 year (AUC 0.63 to 0.68) and 2 years (AUC 0.63 to 0.68), as detailed in Table 5.5.

5.3.4 BNP molecular forms as predictors of death/HF

Cox survival analysis was conducted to investigate the prognostic ability of BNP molecular forms for death/HF at 6 months, 1 year and 2 years, using the same multivariate base model as described for death/MI. NT-proBNP was a univariate predictor of death/HF at 6 months, 1 year and at 2 years ($p\leq0.001$), and retained independent prediction at these end points ($p<0.001$) when added to the base model. BNP molecular forms were also significant univariate predictors of death/HF at 6 months, 1 year and at 2 years ($p<0.001$). When added to the base model, BNP molecular forms retained independent prediction at 6 months ($p\leq0.026$), 1 year ($p\leq0.007$) and at 2 years ($p\leq0.001$) showing comparable prognostic abilities to NT-proBNP (Table 5.4).

Other independent predictors of outcome were age, Killip score >1, STEMI class, systolic BP, β-blockers and statins on discharge and eGFR.

ROC curves were comparable between molecular forms and NT-proBNP for death/HF at 6 months (AUC 0.70 to 0.78), 1 year (AUC 0.70 to 0.77) and 2 years (AUC 0.70 to 0.77), as detailed in Table 5.5.

5.3.5 BNP molecular forms as predictors of MACE

Cox survival analysis was conducted to investigate the prognostic ability of BNP molecular forms for MACE at 6 months, 1 year and 2 years. NT-proBNP was a univariate predictor of MACE at 6 months, 1 year and at 2 years ($p<0.001$), and retained independent prediction at these end points ($p<0.005$) when added to the previously defined base model. BNP molecular forms were also significant univariate predictors of MACE at 6 months, 1 year and at 2 years ($p<0.001$). When BNP molecular forms were added to the model they retained independent prediction at 6 months ($p \leq 0.017$), 1 year ($p \leq 0.002$) and at 2 years ($p<0.001$) showing comparable abilities to NT-proBNP (Table 5.4). Other independent predictors of outcome were age, Killip score >1, STEMI class, revascularisation, systolic BP and β-blockers on discharge.

ROC curves were comparable between molecular forms and NT-proBNP for MACE at 6 months (AUC 0.65 to 0.71), 1 year (AUC 0.65 to 0.70) and 2 years (AUC 0.65 to 0.70), as detailed in Table 5.5.

Table 5.4 Independent prediction abilities of BNP molecular forms using multivariate Cox survival analyses for outcomes of death/MI, death/HF and MACE at 6 months, 1 year and 2 years. Results displayed as HR (95% CI) p value.

	Death/MI	Death/HF	MACE
BNP 5-32			
6 months	1.55 (1.07-2.23), p = 0.019	1.55 (1.05-2.28), p = 0.026	1.46 (1.07-1.99), p = 0.017
1 year	1.62 (1.17-2.24), p = 0.004	1.60 (1.13-2.27), p = 0.007	1.55 (1.18-2.05), p = 0.002
2 years	1.70 (1.26-2.30), p = 0.001	1.78 (1.28-2.47), p = 0.001	1.63 (1.25-2.11), p < 0.001
BNP 4-32			
6 months	1.64 (1.09-2.47), p = 0.017	1.74 (1.16-2.61), p = 0.007	1.55 (1.11-2.18), p = 0.011
1 year	1.67 (1.17-2.39), p = 0.005	1.86 (1.29-2.67), p = 0.001	1.67 (1.23-2.26), p = 0.001
2 years	1.71 (1.22-2.39), p = 0.002	1.96 (1.39-2.75), p < 0.001	1.69 (1.27-2.25), p < 0.001
BNP 3-32			
6 months	1.58 (1.06-2.37), p = 0.026	1.88 (1.25-2.82), p = 0.002	1.63 (1.16-2.29), p = 0.005
1 year	1.54 (1.08-2.21), p = 0.018	1.88 (1.31-2.72), p = 0.001	1.64 (1.21-2.32), p = 0.002
2 years	1.58 (1.13-2.21), p = 0.008	2.06 (1.46-2.91), p < 0.001	1.70 (1.28-2.27), p < 0.001
NT-proBNP			
6 months	1.62 (1.08-2.41), p = 0.019	2.24 (1.40-3.59), p = 0.001	1.72 (1.21-2.45), p = 0.002
1 year	1.50 (1.07-2.12), p = 0.019	2.02 (1.35-3.02), p = 0.001	1.54 (1.14-2.07), p = 0.005
2 years	1.44 (1.06-1.96), p = 0.020	2.15 (1.47-3.14), p < 0.001	1.47 (1.12-1.93), p = 0.005

Models adjusted for: age, sex, PH (past history) MI/angina, Killip score >1, estimated glomerular filtration rate, PH diabetes, troponin (cTnI), ST elevation MI, revascularization, urea, systolic BP, PH hypertension, aspirin on discharge, β-blockers on discharge, ACE/ARB on discharge, statins on discharge

BNP; B-type natriuretic peptide; HF, heart failure; MACE, major adverse cardiac event; NT-proBNP, N-terminal proBNP

Table 5.5 Accuracy of outcome of prediction of BNP molecular forms and NT-proBNP for death/MI, death/HF and MACE at 6 months, 1 year and 2 years showing area under the curve (AUC) for the Receiver Operator Characteristic

	Death/MI	Death/HF	MACE
BNP 5-32			
6 months	0.66 (0.61 - 0.70), $P < 0.001$	0.71 (0.66 - 0.76), $P < 0.001$	0.67 (0.63 - 0.71), $P < 0.001$
1 year	0.66 (0.62 - 0.70), $P < 0.001$	0.71 (0.66 - 0.75), $P < 0.001$	0.67 (0.63 - 0.71), $P < 0.001$
2 years	0.66 (0.62 - 0.70), $P < 0.001$	0.71 (0.67 - 0.75), $P < 0.001$	0.67 (0.63 - 0.70), $P < 0.001$
BNP 4-32			
6 months	0.63 (0.58 - 0.68), $P < 0.001$	0.70 (0.65 - 0.75), $P < 0.001$	0.65 (0.60 - 0.69), $P < 0.001$
1 year	0.64 (0.59 - 0.68), $P < 0.001$	0.70 (0.66 - 0.75), $P < 0.001$	0.65 (0.61 - 0.69), $P < 0.001$
2 years	0.64 (0.59 - 0.68), $P < 0.001$	0.70 (0.66 - 0.74), $P < 0.001$	0.65 (0.61 - 0.69), $P < 0.001$
BNP 3-32			
6 months	0.63 (0.58 - 0.68), $P < 0.001$	0.70 (0.65 - 0.75), $P < 0.001$	0.65 (0.60 - 0.69), $P < 0.001$
1 year	0.63 (0.59 - 0.68), $P < 0.001$	0.70 (0.65 - 0.74), $P < 0.001$	0.65 (0.60 - 0.69), $P < 0.001$
2 years	0.63 (0.59 - 0.68), $P < 0.001$	0.70 (0.66 - 0.75), $P < 0.001$	0.65 (0.61 - 0.68), $P < 0.001$
NT-proBNP			
6 months	0.69 (0.64 - 0.73), $P < 0.001$	0.78 (0.74 - 0.82), $P < 0.001$	0.71 (0.67 - 0.75), $P < 0.001$
1 year	0.68 (0.64 - 0.72), $P < 0.001$	0.77 (0.73 - 0.81), $P < 0.001$	0.70 (0.67 - 0.74), $P < 0.001$
2 years	0.68 (0.64 - 0.72), $P < 0.001$	0.77 (0.73 - 0.80), $P < 0.001$	0.70 (0.66 - 0.73), $P < 0.001$

Results displayed as AUC (95% CI) p value.

BNP; B-type natriuretic peptide; HF, heart failure; MACE, major adverse cardiac event; NT-proBNP, N-terminal proBNP

5.3.6 BNP molecular form comparison against clinical BNP measurements

Clinical BNP concentrations were measured using a point-of-care device (RapidPIA™, Sekisui Medical Co.) in samples that showed detection of BNP 5-32 (n=617).

Univariate analysis showed that clinical BNP was strongly correlated to BNP molecular forms (r_s = 0.676-0.727, p<0.001) and also to NT-proBNP (r_s = 0.646, p<0.001). The levels of correlation are shown in Figure 5.4.

Predictive ability of molecular forms were compared to clinical BNP using Cox proportional hazards regression on the previously described base-model. Results showed that BNP and BNP molecular forms showed similar predictive abilities across the endpoints and time points. BNP 5-32 was superior to the molecular forms and BNP for death/MI at 6 months (p=0.003 vs p=0.006-0.029), 1 year (p=0.004 vs p=0.004-0.022) and 2 years (p=0.003 vs p=0.006-0.039). For death/HF BNP was the superior biomarker showing similar abilities as BNP 3-32 at 6 months (p=0.003), 1 year (p=0.001) and 2 years (p<0.001). Similar prognostic abilities were noticeable between the molecular forms and BNP at 6 months (p=0.009-0.015), 1 year (p=0.002-0.007) and 2 years (p=0.002-0.003) for MACE.

In contrast, NT-proBNP was not able to predict outcome in this subset of patients for time points associated with the end point of death/MI (p≥0.092). For death/HF and MACE, NT-proBNP displayed comparable prognostic qualities as BNP and its molecular forms (p=0.045-0.001). This indicated a modest improvement in predictive ability for BNP and its molecular forms over NT-proBNP. Table 5.6 details the model statistics for all BNP measurements as predictors across all endpoints and outcomes.

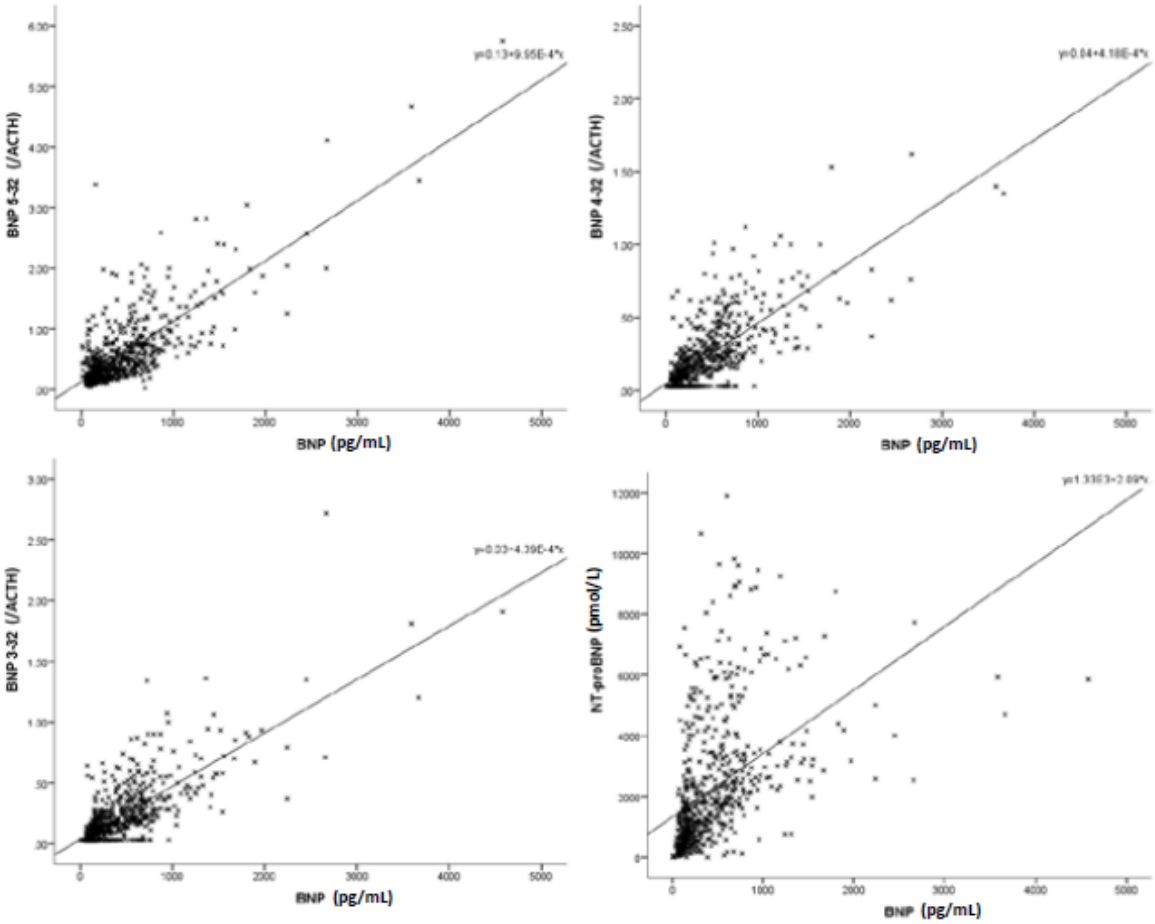


Figure 5.4 Correlations between clinical BNP, BNP molecular forms and NT-proBNP showing a positive correlation (r_s = 0.646-0.727)

Table 5.6 Independent prediction abilities of BNP, molecular forms and NT-proBNP using multivariate Cox survival analyses for outcomes of death/MI, death/HF and MACE at 6 months, 1 year and 2 years in a subset of samples (n=617)

	Death/MI	Death/HF	MACE
BNP			
6 months	2.07 (1.08-3.98), p = 0.029	2.69 (1.41-5.11), p = 0.003	1.98 (1.14-3.43), p = 0.015
1 year	2.12 (1.20-3.72), p = 0.009	2.64 (1.49-4.70), p = 0.001	2.12 (1.30-3.47), p = 0.003
2 years	1.98 (1.17-3.33), p = 0.010	2.77 (1.62-4.74), p < 0.001	2.08 (1.32-3.28), p = 0.002
BNP 5-32			
6 months	2.65 (1.39-5.07), p = 0.003	2.26 (1.22-4.19), p = 0.010	2.03 (1.17-3.50), p = 0.011
1 year	2.27 (1.30-3.98), p = 0.004	2.26 (1.30-3.92), p = 0.004	1.96 (1.20-3.18), p = 0.007
2 years	2.22 (1.32-3.74), p = 0.003	2.48 (1.48-4.15), p = 0.001	2.07 (1.32-3.27), p = 0.002
BNP 4-32			
6 months	2.11 (1.24-3.58), p = 0.006	2.01 (1.20-3.34), p = 0.008	1.78 (1.16-2.75), p = 0.009
1 year	1.92 (1.23-3.01), p = 0.004	2.00 (1.26-3.16), p = 0.003	1.83 (1.25-2.70), p = 0.002
2 years	1.78 (1.18-2.69), p = 0.006	2.01 (1.31-3.09), p = 0.001	1.76 (1.23-2.52), p = 0.002
BNP 3-32			
6 months	1.86 (1.10-3.15), p = 0.022	2.18 (1.30-3.65), p = 0.003	1.80 (1.16-2.78), p = 0.009
1 year	1.69 (1.08-2.66), p = 0.022	2.09 (1.31-3.33), p = 0.002	1.75 (1.19-2.59), p = 0.005
2 years	1.55 (1.02-2.34), p = 0.039	2.20 (1.42-3.40), p < 0.001	1.74 (1.21-2.50), p = 0.003
NT-proBNP			
6 months	1.58 (0.91-2.75), p = 0.107	2.35 (1.25-4.40), p = 0.008	1.63 (1.01-2.63), p = 0.045
1 year	1.45 (0.91-2.30), p = 0.118	2.49 (1.42-4.37), p = 0.001	1.68 (1.10-2.56), p = 0.016
2 years	1.45 (0.94-2.23), p = 0.092	2.50 (1.49-4.20), p = 0.001	1.64 (1.11-2.43), p = 0.014

BNP, B-type natriuretic peptide; HF, heart failure; MACE, major adverse cardiac event; MI, myocardial infarction; NT-proBNP, N-terminal pro B-type natriuretic peptide

5.3.7 Reclassification analysis

To analyse whether BNP molecular forms were able to reclassify or show an improvement in patients with adverse events, reclassification analyses were performed using the continuous net reclassification improvement index by adding each of the molecular forms and NT-proBNP to the current GRACE clinical risk score for outcome at 6 months. Results showed that both NT-proBNP and BNP molecular forms showed a total improvement in reclassification when added to the GRACE score ($P \leq 0.005$) (Table 5.7). In addition to this, BNP 5-32 was able to successfully down-classify risk in patients without an event and up-classify those with an event, providing a successful overall reclassification of patients for adverse event risk ($p<0.001$). Similar findings were noticed for NT-proBNP. In contrast, both BNP 4-32 and BNP 3-32 were able to successfully reclassify overall patient risk ($p=0.005$ and $p=0.003$, respectively), however they were unable to successfully up-classify patients with events (Table 5.7).

5.3.8 Decision tree analysis

To assess the utility of adding BNP 5-32 and NT-proBNP as secondary risk stratification markers after using the GRACE score for risk prediction (death/MI at 6 months), decision tree analysis was performed. Results showed that following the primary use of GRACE, a score >138 and NT-proBNP concentration above 1834.4 pmol/L identified the highest risk group (n=143), with a group event risk of 39.2%. A GRACE score of ≤138 and BNP 5-32 value of ≤0.240 selected the lowest risk group (n=444) with a group event risk of 6.5% showing successful down-stratification of patient risk.

Patients were stratified into tertiles for Kaplan-Meier survival analysis for the outcome of death/MI at 6 months. Results showed a significant increase in risk across the groups ($p\leq0.001$) with a fold-increase in risk of 2.6 and 9.1 for the middle- and high-risk groups when compared to the low-risk reference group ($p<0.001$), respectively (Figure 5.5).

These data suggests the use of NT-proBNP and BNP 5-32 as secondary risk markers after identification by the GRACE score for acute MI.

Table 5.7 Reclassification analyses for all-cause mortality or reinfarction at 6 months using continuous reclassification showing the net reclassification index (NRI) of adding BNP molecular forms and NT-proBNP to the classification using the GRACE clinical risk score

	Endpoint	NRI (95% CI)	p Value
BNP 5-32	No	12.2 (5.3-19.1)	0.001
	Yes	21.2 (4.4-37.9)	0.013
	Total	33.4 (15.3-51.5)	<0.001
BNP 4-32	No	26.9 (20-33.9)	<0.001
	Yes	-0.7 (-17.5-33.9)	NS
	Total	26.2 (8.1-44.3)	0.005
BNP 3-32	No	31.2 (24.3-38.1)	<0.001
	Yes	-.36 (-20.4-13.1)	NS
	Total	27.5 (9.4-45.6)	0.003
NT-proBNP	No	9.8 (2.8-16.8)	0.006
	Yes	30.9 (14.1-47.7)	<0.001
	Total	40.7 (22.5-58.9)	<0.001

95% CI, 95% confidence intervals; BNP; B-type natriuretic peptide; NRI, net reclassification index; NT-proBNP, N-terminal proBNP

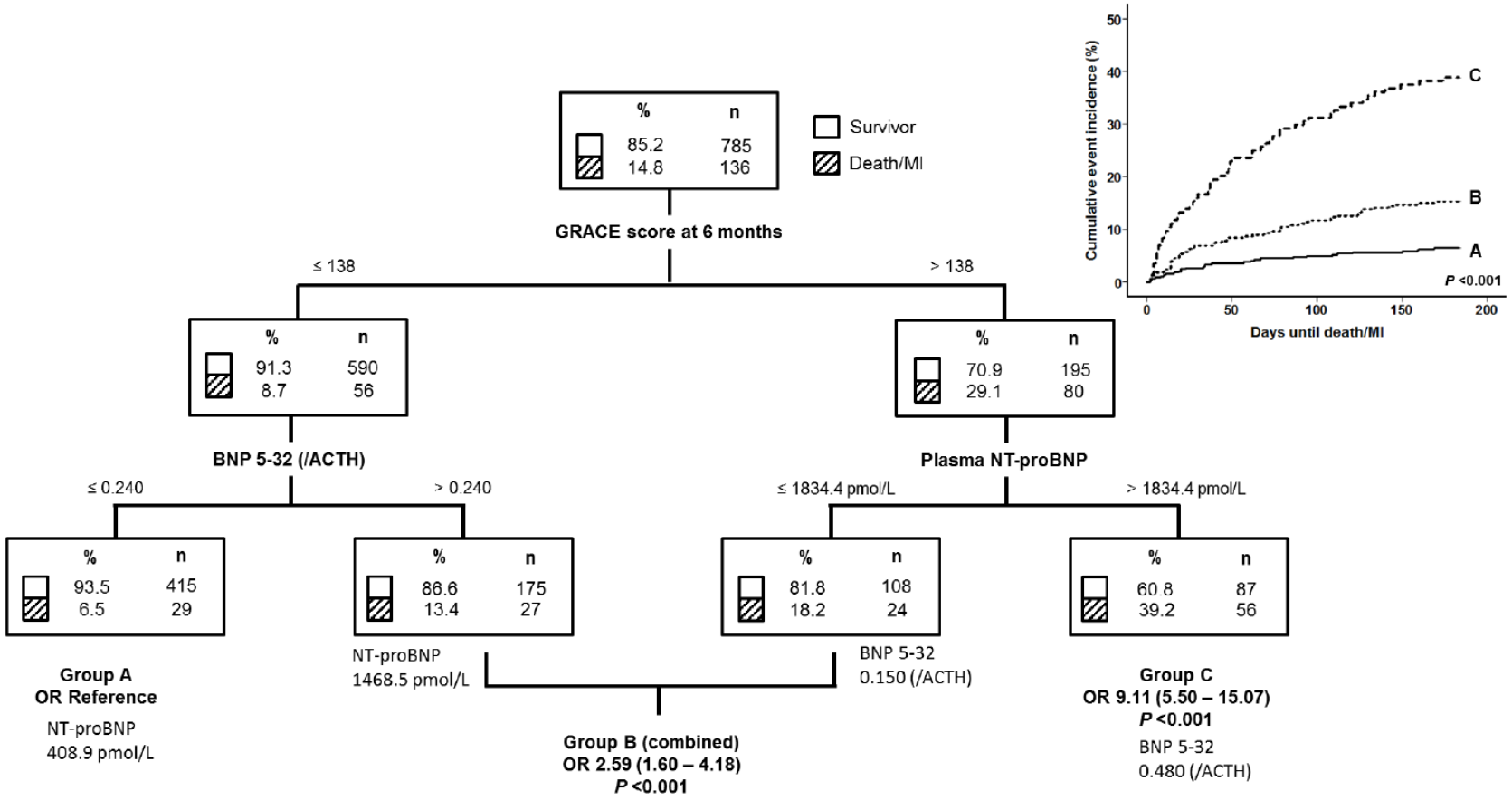


Figure 5.5 Decision tree showing risk stratification for the use of the GRACE clinical risk score as a primary classifier for death/MI at 6 months, followed by secondary risk stratification markers BNP 5-32 and NT-proBNP, and cumulative event incidence of risk groups (inset). For reference, median concentrations of NT-proBNP (pmol/L) (Group A and B) and BNP 5-32 (/ACTH) (Group B and C)

BNP, B-type natriuretic peptide; NT-proBNP, N-terminal proBNP; OR, odds ratio

5.4 Discussion

This study reports that molecular forms of BNP are associated with poor outcome in patients hospitalised with acute MI, and the utility of these molecular forms in clinical risk prediction. The results showed that molecular BNP forms 5-32, 4-32 and 3-32 were all independently able to predict death/MI, death/HF and MACE at 6 months, 1 year and at 2 years after adjustment for traditional clinical and physiological factors. These prognostic qualities were comparable to conventional clinical measurements of circulating BNP through analysis of plasma NT-proBNP concentrations. Although clinical measurements of BNP have been shown as useful in prediction of outcome after acute MI for long-term survival (Omland et al., 1996), this is the first study to report the prognostic capabilities of BNP molecular forms in an acute MI cohort. These findings extend from previous evidence for the utility of BNP molecular forms in acute HF prognosis.

Similarly to results in Chapter 3 (acute HF study), proportional hazards analyses demonstrated that BNP 5-32 exhibited mildly superior risk prediction capabilities when compared to alternative molecular forms. The BNP molecular forms were successfully able to predict outcome at all measured endpoints, with an improvement in prognostic qualities with extended follow-up periods. This suggests that although suitable for shorter-term prediction, BNP molecular forms offer improved information for risk stratification over time when added to the risk model.

Reclassification analyses were performed after combining BNP molecular forms with the established GRACE risk prediction tool for death/MI at 6 months and showed an added benefit for the use of BNP molecular forms. BNP 5-32 was the superior molecular form with the ability to reclassify patients for both increased and decreased risk after classification by the GRACE score and was comparable to results obtained for NT-proBNP. In contrast, although BNP 4-32 and BNP 3-32 were successful in an overall reclassification of patient risk, this was centered on their strong ability to down-classify risk with both molecular forms reporting negative net reclassification indices for the up-classification of patient risk.

BNP 5-32, as the most discriminatory of molecular forms, was taken forward into decision tree analysis and used in conjunction with NT-proBNP to define restratification of risk following GRACE scoring. The GRACE risk score was developed from a multinational registry of over 11,000 patients with ACS. This score is adjusted by eight risk factors for in-hospital death and post-discharge at 6 months and consists of Killip class, systolic blood pressure at presentation, heart rate at presentation, age, creatinine level, cardiac arrest at admission, ST-segment deviation on the index ECG and elevated cardiac enzyme levels. Each risk factor is allocated a different number of points to monitor death (E Backus et al., 2011). After initial GRACE scoring, BNP 5-32 identified patients at lower risk and NT-proBNP identifying those at the highest risk, generating patients groups that showed a stepped increase in risk. Initial GRACE scoring at 138 identified patients in the high-risk category for both non-STEMI and STEMI (GRACE score ≥119 for non-STEMI and ≥128 for STEMI) (Eagle et al., 2004). This data suggests the roles of both BNP 5-32 and NT-proBNP as secondary biomarkers for risk stratification at 6 months after clinical risk scoring. Previous studies have supported the use of NT-proBNP to add prognostic value to the GRACE score in elderly patients with acute MI (Lorgis et al., 2009) and also predict early and late mortality in risk stratification after ACS (Khan et al., 2009).

Clinical BNP was measured in a subset of samples that showed detection of BNP 5-32. BNP showed strong correlations with the molecular forms comparable to those reported by (Miller et al., 2011), supporting previous studies that clinical BNP is composed of its molecular forms and these molecular forms are involved in the degradation of BNP or are products of BNP. Prognostic qualities of BNP molecular forms were comparable to BNP across all time points and endpoints with BNP 5-32 the most superior for death/MI at 6 months, 1 year and 2 years. BNP was more superior for death/HF at 6 months, 1 year and 2 years. However, NT-proBNP was inferior to BNP and the molecular forms in prognosis, certainly for death/MI across all time points and for MACE at 6 months, showing a modest improvement in prognostic ability for BNP molecular forms over NT-proBNP.

5.5 Conclusion

In conclusion, the present study shows circulating BNP molecular forms are associated with a prognosis of poor outcome (death/MI, death/HF and MACE) at 6 months, 1 year and at 2 years. When used in combination with NT-proBNP, BNP 5-32 showed utility as a secondary risk stratification biomarker in identifying low-risk patients for outcome at 6 months after initial categorisation with the GRACE score. This study further supports the potential role of BNP molecular forms in prognosis and risk stratification of acute cardiovascular hospitalizations and warrants further study into the dynamics and kinetics of BNP degradation as well as BNP pathway responses to disease management strategies.

CHAPTER SIX

GENERAL DISCUSSION

6. GENERAL DISCUSSION

This thesis hypothesised that BNP molecular forms would have a potential role in the prognosis and risk stratification in CVD. The prognostic ability of BNP molecular forms have not yet been explored, and thus the nature of research carried out is novel. The implications of this research would be beneficial to clinicians globally and the ability to provide key information for risk stratification and tailored management beyond current guidelines would appeal to clinicians, pharmaceutical companies, and mass spectrometry/analytical companies alike. As CVD is an umbrella term for many diseases, common diseases; acute HF, chronic HF and acute MI where plasma was available were used to implicate this.

The aims of this thesis were to develop a mass spectrometry method to detect BNP molecular forms in acute HF, chronic HF and acute MI. These were then used to assess for prognostic risk prediction and clinical importance.

6.1 Summary of findings

Investigating the prognostic applicability of BNP molecular forms in acute HF showed that BNP molecular forms were associated with a prognosis of poor outcome. This study is the first study to report on the prognosis of BNP molecular forms. Furthermore, this study showed that BNP molecular forms were able to be detected in over 90% of the samples and showed independent prediction qualities of all-cause mortality or a combination of all-cause mortality and rehospitalisation at short- (6 months) and long-term (1 year) when added to a multivariate model adjusted for traditional risk factors. Survival analysis showed that when the molecular forms were split into tertiles, increasing levels of BNP molecular forms were associated with increasing risk of adverse events. The results from this study showed comparable results to clinically measured NT-proBNP.

Out of the molecular forms, BNP 5-32 showed the most superiority for prediction of death and/or death/HF, and also showed the strongest association between elevated levels and reduced survival. For in-hospital mortality, BNP molecular forms were unable to improve risk prediction when added to clinical risk models (ADHERE and GWTG-HF).

Interestingly, BNP molecular forms showed reduced associations with renal markers compared to NT-proBNP, and are suggested to merit in HF risk prediction irrespective of renal function. From this it was understood that BNP molecular forms were detectable using the MS-IA method and showed prognostic abilities in acute HF. It was unclear whether the profiles of the molecular forms were specific to acute HF or whether they displayed abilities in chronic conditions. Furthermore, in the chronic cohort that was available, additional follow-up serial samples were available allowing for analysis of serial measurements post-treatment.

The chronic HF study was a large multicentre study (BIOSTAT-CHF) that aimed to investigate the effects of guideline-based treatment in HF. BNP molecular forms showed prognostic ability at enrolment at short-term (1 year) and long-term (3 year) outcomes. Of the molecular forms, BNP 5-32 was the more superior, and showed comparable prognostic abilities to BNP. Further, as a short-term prognostic marker, BNP 5-32 was more superior to BNP in predicting death/HF suggesting its role in short-term or early prognosis. Furthermore, follow-up analysis showed BNP 5-32 was not responsive to guideline-based treatment suggesting its mechanistic pathway is independent to that of ACEi or beta-blocker, despite BNP responding. The importance of serial measurements for natriuretic peptides has recently been documented, and this study showed that measurement at follow-up was associated with identifying patients at highest risk, irrespective of measurement at enrolment. This supports previous reports on the importance of serial measurements, in particular the influence of follow-up on prognosis. It must be noted that in the overall cohort, only 40% (51% at enrolment and 25% at follow-up) of samples allowed for detection of one or more BNP molecular forms. Compared with 90% detection in the acute HF cohort, the chronic HF cohort suggests that BNP molecular forms lack the sensitivity or detection in chronic cases where the heart's condition is more stable and BNP concentrations are lower. This is certainly evident at follow-up. Lower BNP concentrations in chronic HF would theoretically result in lower BNP molecular forms, however mechanisms of BNP molecular forms in acute and chronic cases are to be investigated.

The first two studies covered the basis of HF; acute and chronic. However, it was important to understand whether BNP molecular forms are able to add important

information prior to HF. Samples from acute MI were available and since MI is the most common cause leading onto HF BNP molecular forms were measured in acute MI samples to investigate their prognostic ability. It was found that as with previous studies, BNP molecular forms were associated with a prognosis of poor outcome at short- (6 months) and long-term (2 years) for death/MI, death/HF and MACE, comparable to NT-proBNP. BNP molecular forms further showed that they were able to reclassify patient risk after combining with the GRACE risk score. As BNP 5-32 was the most superior marker of the BNP molecular forms, a decision tree was constructed and showed that BNP 5-32 was successful in identifying low risk patients after GRACE scoring, whilst NT-proBNP was able to identify high risk.

These studies suggest the potential role of one or more BNP molecular forms in the prognosis of CVD. They showed comparable qualities to current markers and prediction abilities at short and long term.

To note, due to the strong mechanistic relationship (from the same precursor molecule) between BNP, NT-proBNP, BNP 5-32, BNP 4-32 and BNP 3-32, these natriuretic peptide molecules exhibited strong co-linearity. Statistical analysis for all three presented studies showed that when more than one of these biomarkers were added to the same model, their respective influences showed no additional information to the model and the biomarkers were not significant ($p>0.05$).

Furthermore, in the acute HF study, patients where BNP molecular forms were not detected were excluded from further statistical analysis. In contrast, for the BIOSTAT-CHF and acute MI studies, no population was excluded from statistical analysis based on detection or not. It is important to understand that the acute HF study showed more than 90% detection of BNP molecular forms therefore excluding a small proportion of the total population such that the effects on statistical analysis are minimal. However, for the other two studies, detection was less than 50% in the BIOSTAT-CHF study and 57% detection in the acute MI study, therefore excluding undetectable BNP molecular forms in samples would remove a large proportion of the total population and would expect to have significant consequences on the statistical analyses and clinical interpretation of data.

6.2 Review of methods for clinical translation

MALDI-ToF-MS has proven to be a successful method for isolating BNP molecular forms from patient's plasma samples and allowed for successful analysis. Prior to this, the MALDI method has been well documented as a successful diagnostic technology in clinical microbiology allowing for high-throughput, cost effective and efficient output of data (Croxatto et al., 2012, Duncan et al., 2015). For clinical translation in proteomics of patient plasma, continued refinement of the method is required for regulatory approval. The identification and measurement of BNP molecular forms was centred on the use of immunocapture sample preparation with MALDI-ToF-MS analysis. The methods described in this thesis allowed for a maximum of 25 samples to be analysed daily including approximately 4 hours for the immunocapture sample preparation and up to 3 hours of manual mass spectrometry analysis. This method in particular remains an issue for clinical translation due to the low sample throughput. In addition to this, analysis of samples on the mass spectrometer was manually operated for laser positioning and firing to find the 'sweet spot'. This results in the possibility of operator bias as a limitation for the methods used.

In addition to this, there have been attempts to expand the utility of using MALDI for quantitative measurements, however the heterogeneity with the MALDI crystal has posed difficulties at best for this (El-Aneed et al., 2009). Furthermore, using complex mixtures such as biological samples has shown poor reproducibility (sample-to-sample and shot-to-shot) for quantitation due to the so-called sweet spots. Although the issues with the sweet spot are not fully understood, it could be due to the variation in the crystallisation of the matrix. Suppression effects has also shown to hinder quantitative measurements which can distort complex biological samples in a wide concentration range. Due to ionisation efficiencies compounds of a similar nature and dynamic range can affect quantitation due to detector saturation (Szájli et al., 2008).

There have been efforts to quantitate using MALDI and it is desirable to perform quantitation ideally within the sample with isotopically labelled versions of the molecule(s) of interest combating the complex interferences and improving precision and accuracy (Szájli et al., 2008).

In terms of mass spectrometry, the major issues for implementation of mass spectrometry-based clinical assays are its costs and regulatory authority approval to support the methods without extensive testing across institutions. To overcome this, simplified and approved analytical kits, and automated preparations and analysis methods are required to be developed and approved globally to maintain successful cross-validation.

Technologies such as mass spectrometry, which complement traditional enzyme-based assays have allowed the development of highly sensitive and selective methods able to measure multiple biomarkers. However, currently at a stage of infancy for translation to clinical laboratories, these methods offer potential for future advancements. Furthermore, the current interest surrounding multiple omics, including proteomics, metabolomics, lipidomics, and genomics, could lead to the development of a novel panel of biomarkers that provide improved clinical information to patients in CVD and beyond. One example of this is focussing on protein and metabolite markers to improve risk stratification such as that shown by combining BNP/NT-proBNP with trimethylamine N-oxide in acute HF (Suzuki et al., 2016).

6.3 Clinical impact for BNP molecular forms

The premise for the future role of BNP molecular forms in a clinical setting is highlighted in the research conducted in the thesis from acute HF, serial measurement of chronic HF patients, and acute MI cohorts. This advocates the development of a commercial assay that can measure a panel of molecular forms. The future measurement of BNP molecular forms is expected to provide added benefits in CVD prognoses and allow for personalised disease monitoring in patients.

The low interference of BNP molecular forms with renal markers in acute HF may contribute to the superior prognostic value of BNP molecular forms than NT-proBNP, opening a potential avenue of better outcomes based directly on cardiac status. In addition to the findings reported, others have demonstrated the potential of BNP signal peptides in myocardial ischaemia (Siriwardena et al., 2010) and restenosis (Fujimoto et al., 2013). These molecular forms present an exciting and promising role in various

situations and their applications should be further investigated. Clinical ranges of BNP molecular forms remain to be elucidated, and would provide further interesting and valuable information.

From a commercial perspective, it seems reasonable to set up a panel to measure BNP molecular forms (perhaps including BNP, proBNP and NT-proBNP). Such a multi-panel would allow for the exploration of multifaceted details of using BNP molecular forms that go beyond BNP/NT-proBNP for clinical purposes beyond identifying coronary artery restenosis, myocardial ischemia/infarction and assessing prognosis. The multi-panel would allow for a better understanding of the mechanisms and interaction of the molecular forms, interaction of multiple drug effects, or develop better pharmaceutical interventions via manipulation of blood BNP molecular forms levels.

In the era of personalised medicine, the premise for BNP molecular forms to offer additional value in CVD conditions and allow for risk stratification in response to disease management strategies remains to be elucidated.

6.4 Recommendations for future work

6.4.1 Method translation

One of the limitations for this study is the choice of method for clinical translation. Although the MALDI-ToF-MS method has served as a solid method for the detection of these candidate biomarkers, there is room for further development and translation. Firstly, absolute quantitation using this method can be difficult and as discussed in Chapter 6.2, the use of isotopically labelled standards can improve the quantitation, improve precision and accuracy. Secondly, this method allowed for the measurement of 25 samples per day and therefore the lack of throughput is an issue for clinical translation. A method based on a LC-MS triple quadrupole technique would aim to improve both the issue surrounding absolute quantitation and accuracy, and high throughput. Early attempts of this method have shown that LC-MS lacks in sensitivity and further interrogation of the instrument is required. Finally, the limit of detection for this method was in the region of 70 pg/mL BNP 5-32. From the BNP values in the

BIOSTAT study, the chronic patients have presented BNP concentrations below this LOD for BNP 5-32, therefore the limits of this method does not take into consideration the detection of chronic HF patients and certainly not the population of healthy patients.

A prospective workflow of the method development could include the following, but not limited to; direct injection of pure standards of the peptide, standard curve of the peptide from high concentration to minute concentrations to find the initial limit of detection, tweaking the LC method including run time, cone voltage, collision energy, and solvent gradients. This would be followed by attempting to improve the limit of detection by linearising the peptide using DTT and IAA, speedvac and freeze drying to increase sample purity. Once this can be achieved, sample preparation of a plasma sample would include immunoprecipitation, linearisation, solid phase extraction to remove salts, speedvac and freeze dry, before reconstituting the pure sample in formic acid and analysing.

Alternatively, a commonly used ELISA method could also allow for detection of one or more molecular forms. This would involve a secondary antibody specific to a particular peptide and the primary antibody as Sekisui 7. The secondary antibody would be biotinylated and a sandwich ELISA method would allow detection of a particular form of BNP. Early attempts of this method have shown that the method lacks in sensitivity and further interrogation of the instrument and secondary antibody is required.

6.4.2 Statistical interrogation and risk score

Although numerous prognostic markers including BNP molecular forms have been identified for CVD, in particular HF, their clinical usefulness and application is limited and risk stratification remains a challenge. Recently, prognostic risk scores have been developed for HF to predict death, but not hospitalisation. Some risk scores are available as interactive online tools (Rahimi et al., 2014, Ouwerkerk et al., 2014).

Risk score models such as ADHERE and GTWG-HF for HF, and GRACE and Thrombolysis In Myocardial Infarction (TIMI) score used in MI present risk models based on simple clinical and non-clinical variables used for therapeutic decision making such as age,

aspirin use, elevated cardiac markers, angina etc. These are then allocated points for each applicable risk, totalled and interpreted to predict outcomes.

Statistical interrogation using BNP molecular forms for future clinical studies to be used in a risk model to predict outcomes and mortality in a similar fashion accredited to BNP. This could be used in occasions where BNP levels are negligible such as in cases where the new HF medicine, Entresto™, is prescribed.

6.4.3 Sacubitril/Valsartan-The future of ARNI

Sacubitril/valsartan is a novel angiotensin receptor-neprilysin inhibitor (ARNI) recently documented to improve outcomes in patients with HF compared to ACEi (Gori and Senni, 2016). Its mechanisms of actions have not been well defined however its actions cause simultaneous enhancement of the NP system (inducing natriuresis and diuresis) and inhibition of the RAAS combining the effects of Sacubitril and Valsartan (Singh et al., 2017). The direct consequence of this drug is NEP inhibition and an increase in circulating NPs. Such that in the PARADIGM-HF study, BNP concentrations were higher in the sacubitril/valsartan group and presented with improved outcomes compared with enalapril. However, the average BNP increase was small compared with cGMP.

The NEP inhibitor component of the drug is expected to decrease the degradation of BNP into BNP 5-32, thus leading to increased levels of BNP and resulting in difficulties monitoring drug response. This could pave an interesting avenue for BNP 5-32 in the future of ARNI drugs and allow for monitoring of drug response in HF.

6.4.4 Kinetics

Kinetics and mechanistic pathways of BNP molecular forms are not fully understood, although reports suggest that numerous proteases are responsible. Hypothesis-driven proteases investigated suggest to have distinctive degradation pathways, including dipeptidyl peptidase-IV (BNP 3-32) and a neutral endopeptidase (BNP 5-32) processing of BNP 1-32 and a corin-mediated cleavage of proBNP (BNP 4-32), as discussed in Chapter 1.4.7.

However, it is not currently known whether a common distribution of BNP molecular forms is patient-specific or disease-specific. BNP 5-32 has shown superiority over BNP 4-32 and BNP 3-32, however the distribution in a range of diseases is unknown. Investigating this would allow a better understanding of the mechanisms of action for the molecular forms specifically for each disease allowing for tailored therapeutic interventions. In addition to this, the bioactivity of the molecular forms are unknown in vivo and the data is not well established for cGMP activity. Jessica O'Rear et al. investigated the bioactivity of a few proteolysed BNP peptides, including BNP 4-32, BNP 5-32, BNP 5-31, BNP 1-25, and BNP 1-26, and found no differences compared with BNP 1-32 (unpublished data). Clarification of this would allow for further understanding of the roles of BNP molecular forms in natriuresis and diuresis.

One proposed study could include spiking BNP molecular forms into plasma containing different enzymes, inhibitors and promoters and measuring BNP molecular forms at different time points. This would allow for better understanding of the responsible enzymes for each molecular form's degradation. Mimicking different CVD states and spiking different BNP molecular forms would also allow for understanding the natriuretic peptide effects compared to BNP by measuring cGMP activity.

6.5 Concluding remarks

The research presented in this thesis describes investigations into the utility of BNP molecular forms as a new generation biomarker for the prognosis of HF and MI. This initial report showed BNP molecular forms as potential prognostic markers for CVD. The findings are of interest, but require further experiments and developments. This thesis has investigated the use of BNP molecular forms in two domains: i) the applicability of mass spectrometry as a technology in future medicine; ii) clinical impact of BNP molecular forms as viable biomarkers to aid the prognosis of CVD extending beyond current clinical biomarkers.

Following earlier research that showed that due to the short half-life of intact BNP, clinical BNP is the combination of many BNP molecular forms, the research presented in this thesis investigated the prospect of BNP molecular forms as biomarkers in CVD.

The common forms detectable by mass spectrometry were BNP 5-32, 4-32 and 3-32 formed from the removal of 4, 3 and 2 amino acids respectively. The data presented showed that BNP molecular forms, as with BNP, showed a prognosis of poor outcome with elevated levels in acute HF, acute MI, and chronic HF. Furthermore, it showed how serial measurements were able to govern the outcome and risk prediction in a chronic HF cohort suggesting the importance of serial measurements in clinics for improved risk management. Finally it describes a suitable mass spectrometry technique for proteomic analysis.

The advancement of this research would progress this field by offering a potential prospect of a multipanel of cardiac-specific biomarkers, that when combined with risk factors provide risk monitoring. The use of a multi-panel in clinics would allow for better understanding of the underlying mechanisms and response to tailored drugs treatment or the development of better pharmacological interventions by altering plasma BNP levels. Personalised medicine could go beyond the single biomarker analysis and allow for a multi-omics approach such as that shown by the combination of NT-proBNP/BNP with trimethyl N-oxide (TMAO), a metabolic marker linked to gut microbial breakdown of dietary molecules (Suzuki et al., 2016). The use of mass spectrometry as a quantification tool in future medicine broadens the complexity of treating patients through its use of allowing for specific multi-panel and multi-omics approaches using limited assays. The research conducted is not without its limitations and difficulties, but the current data described shows impactful steps towards a new era for personalised medicine.

6.6 Conclusion

The research demonstrates, for the first time, that BNP molecular forms show prognostic ability comparable to clinically measured BNP in HF and MI. They also show usefulness as secondary markers in addition to a risk score, and the ability to monitor patients. Further research is warranted for clinical translation, however, these initial studies provide a hope for novel biomarkers in CVD and mass spectrometry, so that today's techniques can be employed and become common practice in the future.

CHAPTER SEVEN

REFERENCES

7. REFERENCES

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