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Biomarkers of neurohormonal activation, on-going myocardial damage, haemostasis and inflammation in patients with stable chronic heart failure due to left ventricular systolic dysfunction and potential novel treatment

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Abstract

The prognosis of patients with chronic heart failure (CHF) due to left ventricular systolic dysfunction (LVSD) remains poor despite the progress in modern therapy. B-type natriuretic peptide (BNP) is a useful diagnostic and most consistent prognostic markers in CHF. However, treatment strategy guided by natriuretic peptides does not necessarily improve the outcome. In addition to myocardial remodelling, CHF is a systemic syndrome involving neurohormonal activation, inflammatory up-regulation, endothelial dysfunction and haemopoietic, haemostatic and haemorrheologic disturbance. In patients with CHF, the projects of this thesis investigated the potential prognostic role of some biomarkers which reflect these pathophysiological processes in addition to NT-proBNP.

The haemostatic markers investigated were D-dimer and fibrinogen for thrombogenesis; t-plasminogen activator and plasminogen activator inhibitor-1 for fibrinolysis, von Willebrand factor activity and soluble E-selectin for endothelial function, and soluble P-selectin for platelet activity. The role of white and red cell variables from routine full blood count was also explored. Heart-type fatty acid-binding protein (H-FABP), a sensitive marker for myocardial injury, was used as a marker for on-going myocardial damage/remodelling. The change in the level of these markers with time for dynamic risk stratification was also explored.

Coronary artery disease (CAD) is the commonest cause of CHF and conventional invasive revascularisation has not been proven to improve the prognosis of the patients. Enhanced external counterpulsation (EECP) has been shown to improved myocardial perfusion mainly in patients with CAD. Building on the experience from studies of patients with angina, potential role of EECP in improving myocardial function and perfusion in patients with LVSD and CAD was investigated. Its effects on some biomarkers were also investigated.

These studies confirmed the potential prognostic value of a few laboratory markers including H-FABP; whilst showing that EECP can improve regional myocardial function and perfusion with a short-term reduction in H-FABP.

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Declaration

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Chapter 1 Intoduction and literature review

Biomarkers of neurohormonal activation, ongoing myocardial damage, haemostasis and inflammation in patients with stable chronic heart failure due to left ventricular systolic dysfunction and potential novel therapy

1.1 Introduction

The prognosis of patients with chronic heart failure (CHF) due to left ventricular systolic dysfunction (LVSD) remains poor despite the vast progress in modern therapy within the last two decades. Substantial progress has also been achieved in stratification of the risk of CHF patients, especially in the use of laboratory tests and biomarkers such as brain natriuretic peptides (BNPs). However, it remains unclear if treatment strategy guided by these biomarkers can improve the prognosis of patients with CHF. Biomarkers associated with the various pathophysiological processes of CHF may improve risk stratification and help to target appropriate treatment options in these patients. Therefore, we investigated the potential prognostic role of heart-type fatty acid-binding protein (H-FABP), a sensitive marker for myocardial injury, and biomarkers of haemostatic abnormality in addition to the more established biomarkers such as BNPs and high-sensitivity c-reactive protein (hs-CRP) in patient with CHF. The risk of CHF patients may also change with time and measuring the change in the level of these markers with time for dynamic risk stratification may be a better way in assessing the risk of these patients. We also investigated the value of red and white cell variables that are easily available on full blood count and blood films.

1.2 Background

Despite modern heart failure treatment, the prognosis of patients with CHF due to LVSD remains poor and the 3-year mortality is approximately 25%.[1] In recent years, focus in identifying new therapies or strategies such as the use of aldosterone antagonists in post-myocardial infarction (post-MI) patients or in those with milder CHF symptoms[2,3], cardiac resynchronisation therapy in symptomatic patients[1] and ivabradine in those with heart rate above 70 beats per minute[4] have helped improve the prognosis of CHF patients. The advances in technology such as telemonitoring[5] and

thoracic impedence monitoring[6] has also revolutionalised the way these patients are being monitored with a positive impact on their prognosis. A few biomarkers such as BNPs, troponins and high-sensitivity c-reactive protein (hs-CRP) can help to stratify the risk of CHF patients in addition to some of the conventional risk markers such as age, New York Heart Association functional classification (NYHA), severity of LVSD, renal dysfunction and anaemia. More recently, red cell distribution width has been found to be an independent predictor of prognosis in CHF and has incremental prognostic value over NT-proBNP.[7] Therapeutic strategy guided by the change in BNP level has improved the treatment optimisation in CHF patients but it remains unclear if such strategy is better than conventional clinical practice.[8] Newer biomarkers focus on the pathophysiological processes of CHF and /or multi-marker approach and serial measurement of all markers for dynamic risk assessment may help to improve risk stratification in these patients and guide the therapeutic strategy.

CHF is a systemic syndrome that does not only involve myocardial remodeling but is also associated with neurohormonal activation, up-regulation of the inflammatory response, endothelial dysfunction and disturbed haemostasis, haemorheology and haemotopoiesis. Collectively, these processes play a role in the progression of the heart failure syndrome and may be related to the morbidity and mortality.

1.2.1 Ongoing myocardial damage

Irrespective of the aetiology of CHF, progressive myocyte loss may contribute to the progression of myocardial dysfunction.[9,10] This process may be active even in clinically stable patients and is suggested by the detection of cardiac enzymes or myofibril proteins in the plasma. Patients with CHF have minimally raised serum troponins even in the absence of overt myocardial ischaemia or obstructive coronary disease[11] and those with raised troponin T (TnT) had patchy fibrosis and degenerative changes in the heart at post-mortem.[12] These observations gave rise to the concept of ongoing myocardial damage. The mechanism of troponin release in CHF is unclear but likely due to multiple pathophysiological processes. Increased left ventricular wall stress in LVSD can lead to an impaired regional myocardial flow reserve in the absence of significant coronary disease and increased myocardial oxygen consumption, hence subclinical ischaemia and abnormal myocyte metabolism can occur.[13] Others such as apoptosis, neurohormonal activation and inflammation may also involved.[14,15] Raised

serum troponins T and I (TnT and I) in patients with CHF is associated with a worse prognosis.[11,16,17] Combined measurement of cardiac TnT and amino-terminal pro-brain natriuretic peptide (NT-proBNP) improves risk stratification in patients with CHF.[18]

More recently, H-FABP, a cytosolic protein which is released rapidly from injured cardiomyocytes even without myofibril damage has been shown to be more sensitive than troponins in the early diagnosis of acute coronary syndrome and confers prognostic information in the absence of raised troponins. [19-21] In patients with decompensated heart failure, raised H-FABP is more likely to be detectable than a raised troponins suggestive that H-FABP may be more sensitive than troponins in detecting ongoing myocardial damage in these patients.[22] Many studies have shown that a higher level of H-FABP is associated with a worse prognosis in patients with heart failure and may have prognostic value in addition to BNP.[22-31] However, the majority of these studies focused only on a selected group of patients suffering from decompensated heart failure. The role of H-FABP in patients with stable CHF due to LVSD remains unknown.

1.2.2 Perturbed haemostasis and endothelial dysfunction in CHF and LVSD

LVSD is associated with a hypercoagulable state due to factors classically known as the Virchow's Triad.[32] Poor LV contractility, dilated heart chambers with or without LV aneurysm, reduced cardiac output, co-existing atrial fibrillation and physical inactivity with associated venous stasis lead to abnormal blood flow. The resultant abnormal shear stress in combination with neurohormonal activation and inflammatory response cause endothelial injury and dysfunction (ie. Abnormal vessel wall). Consequently, platelet activation, coagulation cascade activation, increased plasma viscosity and impaired fibrinolysis generate a prothrombotic blood constituents.[33,34]

The reported incidence of thromboembolism in patients with CHF due to LVSD from observational studies varies between 0.9 – 42.4 events per 100 patients year mainly due to the difference in patient population.[35-38] From the data of larger-scale randomised controlled trial of CHF, the annual incidence of stroke, peripheral thromboembolism and pulmonary embolism is between 1.2 – 2.3%, 0.1 – 0.3% and 0.2 – 0.3% respectively.[39-44] In Warfarin and Antiplatelet Therapy in Heart failure Trial (WATCH) which randomised 1587 CHF patients who were in sinus rhythm to aspirin,

clopidogrel or warfarin therapy with a mean follow-up of 1.9 years, the incidence of strokes was 2.3%, 2.3% and 0.6% respectively, systemic embolism was 0.8%, 0.8% and 0.4% respectively and pulmonary embolism was 0.4%, 0.2% and 0.2% respectively.[45] In Wafarin/Asprin Study in Heart failure (WASH) trial, 289 CHF patients were randomised to placebo, aspirin or warfarin therapy, the incidence of stroke was 2.0%, 2.2% and 0% after a mean follow-up of 27 months.[46] The incidence of stroke increases by 18% with every 5% point reduction in the left ventricular ejection fraction (LVEF).[39]

Thromboembolism is associated with significant morbidity and mortality in CHF patients. In EPHESUS study with a mean follow-up of 16 months, stroke was the cause of death in 26/407 and 28/483 deaths and also the cause of hospitalisations in 70/606 and 54/649 patients who had one or more cardiovascular hospitalisation randomised to eplerenone and placebo respectively.[2] However, many thrombotic events can be clinically silent. In a post-mortem study of 131 patients with idiopathic dilated cardiomyopathy, 79 (60%) had clinical or autopsy evidence of pulmonary or systemic embolism.[47] Of them, 25% had evidence of pulmonary or systemic emboli identified only at autopsy whereas 30% had clinically documented embolic events without concurrent autopsy finding. Alarmingly, in the 52 patients who did not have clinical or autopsy finding of embolic events, 36 (69%) of them had intracardiac thrombus or plaque.[47]

However, there is little evidence from RCT that anti-thrombotic therapy can reduce the thromboembolism or improve prognosis in CHF patients with or without atrial fibrillation. Indeed, data from WASH and WATCH studies suggest that aspirin may increase the risk of hospitalisation due to decompensated heart failure when compared to warfarin in these patients.[45,46,48] However, both these study were terminated early due to slow recruitment. The HEart failure Long-term Antithrombotic Study (HELAS) randomised 197 patients with CHF due to ischaemic heart disease (IHD) to aspirin or warfarin and those with dilated cardiomyopathy (DCM) to placebo or warfarin.[49,50] This study found a low thromboembolic event rate and anti-thrombotics did not affect the outcome of these patients.[49,50] Warfarin versus Aspirin in patients with Reduced Cardiac Ejection Fraction (WARCEF) study suffered from similar problem with slow recruitment rate but the results have recently been reported. The study shows no net benefit when compared warfarin to aspirin in patients with CHF and in sinus rhythm.[51] However, younger patients may benefit from warfarin therapy and warfarin may be beneficial in reducing the risk of cardioembolic ischaemic stroke based on post-

hoc subgroup analysis.[52,53] Therefore, patient selection may be the key issue when considering antithrombotic therapy in patients with CHF and in sinus rhythm.

The interactions between haemostasis, endothelial function, inflammation and neurohormonal activity are complex and not fully understood.[54,55] Many biomarkers for haemostasis and endothelial dysfunction have been found to confer prognostic value in patients with CHF.[34] D-dimer (DD), a marker of thrombus formation, may have an incremental prognostic value over BNP or hs-CRP.[56,57] However, studies investigating the interaction and prognostic value of these biomarkers in patients with CHF were small and/or consisted of selected group of patients with acute decompensated heart failure or a mixture of systolic and diastolic dysfunction and most reports focused on only a few aspects of the interactions. [56-60]

1.2.3 Inflammation and CHF

The concept of inflammatory response associated with CHF was first documented in 1956 when Elster and co-workers demonstrated that CRP was positively correlated to the severity of CHF.[61] The interest was only revitalized when Levine et al demonstrated a raised tumour necrosis factor alpha (TNF-α) in patients with severe CHF.[62] Later, the 'cytokine hypothesis' was proposed as one of the mechanisms of heart failure.[63] It is now accepted that the progression of heart failure is, at least in part, the result of cardiac and systemic effects caused by immune activation mediated by various cytokines.[10,64]

The most commonly implicated cytokines are TNF-α, interleukin-1 and 6 (IL-1 and IL-6). These cytokines are mainly secreted by mononuclear cells and myocardium.[65] The release of these cytokines is induced by myocardial injury, peripheral tissue hypoxia due to underperfusion and the effects of catecholamine on myocardium.[65] As the proposed endotoxin-lipoprotein hypothesis,[66] bacterial endotoxins translocated through the edematous bowel wall are also potent stimulators for cytokine release in CHF. Cytokines can affect the myocardium leading to LV dysfunction, myocardial remodeling, cardiomyocyte apoptosis and b-receptor uncoupling.[65] Cytokines also have multiple systemic effects including endothelial dysfunction, insulin resistance, inducible nitric oxide synthase (iNOS) activation and its related actions and anorexia and/or cachexia.[65]

A few pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 have prognostic value in patients with CHF but their assays are not easily available and are often expensive and/or require specialised laboratory techniques. On the other hand, hs-CRP is more widely available and relative cheap to measure, making it an excellent biomarker for inflammation. hs-CRP is released from liver in response to stimulation by pro-inflammatory cytokines and therefore closely related to immune activation in heart failure. The prognostic value of CRP or hs-CRP in patients with CHF has been well investigated and may be incremental to that of BNP and troponins.[67-69]

Although immune activation plays a major role in the progression of heart failure and proinflammatory cytokines confer prognostic information, immunotherapy such as etanercept and infliximab do not alter the clinical course or prognosis of patients with CHF due to LVSD.[70,71] Nevertheless, risk stratification using hs-CRP may help target treatment to the appropriate patient population. An example is the identification of a group of CHF patients with higher hs-CRP who may benefit from rosuvastatin treatment in the CORONA Study.[71]

1.3 Blood film variables

1.3.1 Red cell variables

1.3.1.1 Anaemia

Anaemia is common in CHF with a prevalence ranging from 7 – 50% depending on the study population and defining criteria.[72] It is generally defined as Hb < 13 g/dL in men and < 12 g/dl in women according to the WHO Classification. It is more common in women, the elderly and those with lower body weight, renal impairment, greater inflammatory response and more advanced disease status.[73,74] Anaemic CHF patients also have greater morbidity and mortality with more symptoms, worse functional status, more severe LVSD, higher risk of heart failure hospitalisation and reduced survival.[75] The pathophysiology of anaemia in CHF remains unclear but it is often normochormic and normocytic without the classical haematinic deficiency.[76] Multiple pathogenetic mechanisms have been proposed.[74,77]

In some patients, haemodilution due to plasma volume expansion may explain the apparent anaemia. This is more common in those with more advanced disease and is associated with a worse prognosis.[78]

More than a third of CHF patients are deficient in one or more of the classic haematinics[73]; whilst up to 37% alone may have iron deficiency that has been found to be an independent predictor of a worse prognosis.[79] CHF patients are predisposed to iron deficiency due to malabsorption, increased macrophage iron storage, chronic occult gastrointestinal blood loss and proteinuria in those with renal impairment.[74] Indeed, intravenous iron supplement (iron sucrose in FERRIC-HF and ferric carboxymaltose in FAIR-HF trials) can improve the short-term quality of life and functional status of CHF patients especially in those who are anaemic.[80,81]

Renal erythropoietin (EPO) production is also impaired in patients with CHF. Although EPO levels are elevated in CHF patients[82], it may be inadequate relative to the demand and the degree of renal hypoxia. Chronic kidney disease is also common in patients with CHF[73], thus leading to inadequate EPO production. EPO production may also be blunted by inflammatory cytokines due to up-regulation of inflammatory response in CHF.[83] Animal model has provided direct evidence of suppressed renal EPO production by inflammatory cytokines.[84] In addition, treatment with angiotensin converting enzyme inhibitors (ACEIs) may also inhibit endogeneous EPO production.[82]

On the other hand, inflammatory cytokines, especially TNF, has been shown to interfere with the peripheral actions of EPO leading to EPO resistance.[85] Inflammatory cytokines can also disrupt appropriate erythropoiesis and desensitise bone marrow erythroid progenitors to EPO, blocking its anti-apoptotic and pro-maturation effects.[86] In animal study, induction of heart failure was associated with attenuation of pro-erythroblast population by ~40% and proliferative capacity by ~50%. A 3-fold increase in pro-erythroblast destruction was also observed and this correlated to the increase in TNF-mediated apoptosis.[87] Interestingly, the serum of anaemic CHF patients treated with an ACEI can inhibit the proliferation of erythropoietic progenitor cells.[88] This was found to be associated with lower ACE and higher N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), an inhibitor of haematopoiesis which is almost exclusively metabolised by ACE.[88] Therefore, future studies will continue to contribute to our understanding of the pathogenetic mechanisms of anaemia in CHF.

Silverberg et al. was the first to introduce the concept of correcting anaemia in order to improve the clinical outcome of patients with CHF. Although intravenous iron

therapy can improve the functional status of patients in the short term, the role of EPO supplement remains to be investigated. In the STAMINA-HeFT study, darbepoetin increased the Hb in anaemic CHF patients but it did not improve their functional status or prognosis.[89] The on-going RED-HF study may provide more information in due course.[90] Nevertheless, some have postulated that anaemia may indeed be a defense strategy, for example, to reduce iron availability to invading pathogens or to reduce plasma viscosity and coagulability.[77]

1.3.1.2 Red cell distribution width (RDW)

Red cell distribution width (RDW) is a numerical measure of the variability in the size of circulating erythrocytes (anisocytosis). It is measured during the standard full blood count test. Conventionally, a raised RDW is associated with iron deficiency. Disturbed or ineffective erythropoiesis and increased erythrocyte destruction cause heterogeneity in the erythrocyte size and hence a higher RDW. Men and African Americans have higher than women and white Caucasians.[91]

RDW has been found to be directly associated with the prognosis of patients with a variety of non-cardiovascular and cardiovascular conditions. It is also an independent marker for future cardiovascular outcome in the general population[92] or healthy low risk population.[91,93] In a community-based study, RDW was not only predictive of cardiovascular mortality but also death from cancer and respiratory disease.[92] It is an independent prognostic marker or a marker for severity of disease process in patients with hepatitis[94], inflammatory bowel disease[95], haematological malignancies[96], other solid cancers[97], chronic obstructive airway disease[98], pneumonia[99], idiopathic pulmonary hypertension[100], pulmonary embolism[101] and etc. RDW is also a prognostic marker for hospitalised patients[102,103] and patients who are critically ill[104], who suffer from septic shock[105] or those who had out-of-hospital cardiac arrest[106].

RDW is also a strong predictor of morbidity and mortality in unselected man referred for coronary angiogram,[107] patients with stable coronary artery disease (CAD),[108] a prior ST elevation myocardial infarction (STEMI) without CHF[109] or following percutaneous coronary intervention (PCI)[110,111] and in patients with stroke.[112] RDW is also a prognostic marker following non-ST elevation MI (NSTEMI) or unstable angina (UA),[113] STEMI[114] or primary PCI (PPCI) for STEMI.[115] In patients

underwent PPCI for STEMI, higher RDW was associated with greater incidence of no reflow phenomenon.[116,117]

In a population-based study in Malmo, Sweden, higher RDW has been found to be associated with the development of first HF episode requiring hospitalisation.[118] In patients admitted with acute decompensated HF (ADHF), higher RDW is also associated with a worse longer term prognosis independent of BNP or Hb.[119-121] Following treatment, an increase in RDW prior to or 1 month following hospital discharge also confer a worse prognosis independent of BNP levels.[121,122]

Felker et al. first reported the prognostic value of RDW as a predictor of mortality and HF hospitalisation in CHF patients with LVSD or preserved systolic ejection fraction (PSEF) who were enrolled into the CHARM studies.[123] However, Al-Najjar et al. was the first to show that in a large cohort of patients with CHF due to LVSD, RDW conferred incremental prognostic value over NT-proBNP.[7] In the study, RDW was more powerful than and independent of Hb in predicting the long-term mortality. The usefulness of RDW in stratifying the risk of CHF patients was later validated by other studies.[124-127] Dynamic risk stratification can also be accomplished by serial measurement of RDW. An increase in RDW over a 12-month period is independently associated with a higher mortality in seemingly stable ambulatory CHF patients.[128]

In addition to prognosis, higher RDW is also related to increased LV filling pressure,[129] impaired exercise tolerance[130] and impaired reverse remodelling or poor response to CRT.[131,132] It has been postulated that the same pathogenic mechanisms that lead to anaemia also contribute to the increase in RDW. In 195 patients referred to a heart failure clinic with LVEF < 45%, Forhecz et al. found that RDW correlated to markers of neurohormonal activation, inflammation, ineffective erythropoiesis, nutritional status and renal function.[127] Iron deficiency was the strongest determinant of a high RDW in this study. Intuitively, RDW may be a good integrative marker of these processes.

It is unclear how increased RDW can be associated with higher cardiovascular risk in healthy subjects, general population and those with cardiovascular or non-cardiovascular diseases. One recent study found a modest positive correlation between RDW and total cholesterol erythrocyte membrane (CEM) levels[133]. CEM can affect the size and shape of erythrocytes. Higher CEM is associated with unstable coronary artery disease and acute coronary syndrome.[133] Whether this may partly explain the

association between RDW and cardiovascular risk awaits further studies. It is also unknown if treatment of CHF reduces RDW and whether RDW can be a useful tool in guiding CHF therapy.

1.3.2 White cell variables

White cell differential count is also readily available as part of the routine full blood count test. White cell count (WCC) and its subtypes are classic markers of inflammation in cardiovascular disease.[134] Various studies, including population-based epidemiologic data, have shown that increased WCC is associated with a higher incidence of MI and stroke[135-138] and a worse prognosis in patients with coronary artery disease.[139] In the Malmo Preventive Project that screened 22,444 men for detection of individuals with high-risk for cardiovascular disease, 16,940 of the participants with a mean age of 44 years were without history of MI or stoke. These men were followed for over 23 years and the incidence of HF was higher in those with higher WCC.[140]

Most studies that investigated the prognostic value of white cell variables in heart failure involved only patients with decompensated heart failure[141,142] and/or did not include BNP[143-147] or red cell variables[148,149] A raised white cell count (WCC) and lower relative lymphocyte count (RLC) are also related to a worse prognosis in patients with stable or acute decompensated heart failure (ADHF).[7,142,143,148] Neutrophil-to-lymphocyte ratio (NLR), a potent marker for inflammation, has also been shown to be predictive of long-term mortality in the patients admitted with ADHF but its prognostic value in CHF patients is unknown.[141]

In a retrospective analysis of the Studies of Left Ventricular Dysfunction (SOLVD),[148] WCC and neutrophil count (NEC) but not absolute lymphocyte count were independent predictors for long-term all-cause and cardiovascular mortality in patients with CHF on stable medications. However, BNP and red cell variables were not included in the analysis. The same study found that the predictive value of WCC was only applicable to the ischaemic CHF patients. In contrast to the analysis on SOLVD,[148] a retrospective analysis performed on Valsartan Heart Failure Trial (Val-HeFT) with the inclusion of BNP has showed that NEC and absolute lymphocyte count were independent predictors of death and morbid events in CHF patients.[149] However, red cell variables were not included in the model. A lower RLC has also been shown to be an independent predictor of a worse prognosis and is incorporated into the Seattle Heart

Failure Model for prediction of survival in patients with CHF.[150] Nevertheless, the Seattle model does not include BNP.

Leukocyte redistribution is a known phenomenon in patients with CHF. Compared to normal subjects, patients with CHF have the same level of WCC but higher NEC and lower lymphocyte counts regardless of the aetiology of LVSD.[151,152] It is more apparent in patients with more severe CHF[143,148] or in those who are not taking a β-blocker.[151] The pathophysiology of relative neutrophilia and lymphopenia in CHF is not well understood nor it is clear if this is a mere consequence of CHF syndrome or whether it plays a part in the progression of CHF. The neutrophils in patients with CHF are activated[153] and have increased lifespan due to a reduction in apoptosis.[154] On the other hand, increased sensitivity to cytokine-induced apoptosis and redistribution from peripheral blood to other sites have been postulated as the key mechanisms for relative lymphopenia.[155] The T helper and B cells are the main cell types affected although cytotoxic T cells are also lower in CHF patients especially in those who are not taking a β-blocker.[151] The activity of natural killer cells[156] and T-suppressor cells[157] is also reduced in patients with CHF.

CHF is associated with chronic inflammatory response[158] which may modulate the redistribution of leukocytes. However, there is only moderate correlations between white cell variables and hs-CRP or inflammatory cytokines suggestive that processes other than inflammation may also be involved.[144,151] CHF is also associated with sympathetic activation and chronic activity on the β-adrenergic receptors can cause desentitisation and inhibition of lymphocyte proliferation[159,160], whilst increases neutrophil proliferation.[161] Although leukocyte redistribution in CHF may also be a direct response to physiological stress, no correlation was found between some white cell variables and cortisol level.[144]

Activated neutrophils release a wide range of proteolytic enzymes such as myeloperoxidase which is associated with abnormal myocardial remodeling and also the progression of heart failure.[162] Relative lymphopenia may increase predisposition to infection which is a common precipitating factor for decompensated HF and cause of death in patients with CHF.[163] However, anti-cytokine therapy not only ineffective in improving the outcome but may potentially be harmful to patients with CHF.[164,165] Whether more complex immunomodulation therapy aims at preventing neutrophil

activation and lymphocyte apoptosis is a potential therapeutic target in patients with CHF remains to be investigated.

1.4 Amino-terminal of pro-B-type natriuretic peptide (NT-proBNP)

BNP or the amino terminal of its precursor (NT-proBNP) are the most potent diagnostic and prognostic biomarkers of heart failure, in both patients with acute decompensation or chronic stable state due to either LVSD or PSEF.[166-168] BNP-guided therapy may improve the outcome of CHF patients as it encourages more aggressive use of angiotensin converting enzyme inhibitors (ACEIs) and β-blockers. However, it remains a debate whether treatment strategy guided by BNP may confer better outcome than a more conventional clinical approach.[8] A recent meta-analysis of 8 randomised controlled trials (RCTs) comparing BNP-guided therapy and conventional management approach in patients with CHF has shown that BNP-guided strategy reduces all-cause mortality especially in patients younger than 75 years. However, it does not reduce all-cause hospitalisation in these patients.[169,170] Another potential aspect of biomarker-guided therapy is elucidated in the post-hoc analysis of CORONA study where rosuvastatin was found to be beneficial only in patients with lower NT-proBNP.[171] Therefore, using other biomarkers in addition to BNP may improve risk stratification, guide physicians to target the therapy and possibly improve the prognosis of patients with CHF.

1.5 c-reactive protein and high-sensitivity c-reactive protein (hs-CRP)

CRP was first discovered in 1930. It is an acute phase protein that is synthesized exclusively by the hepatocytes in response to pro-inflammatory cytokine stimulation and released into circulation within 6 hours of the stimulus. The level can increase 100-fold within 24 to 48 hours. Although not fully understood, CRP is not only a marker of inflammation but also plays a role in inflammatory process and acts synergistically with cytokines such as augmenting IL-1β induced iNOS production.[54,65] CRP involves in opsonisation and removal of membrane and nuclear material from necrotic cells. It binds to complement factors C1q and H leading to activation of classical pathway of complement system.[65] CRP can also up-regulate the expression of cell adhesion molecules on endothelium.[172]

Elster and co-workers first demonstrated that CRP was positively correlated to the severity of CHF in 1956.[61] But the involvement of CRP in the pathogenesis and progression of heart failure and its prognostic value in CHF patients have only been more extensively investigated since the 90's.[67] Newer high-sensitivity assays have also been developed to detect lower CRP levels with a low detection limit of 0.1 – 0.2 mg/L.[173] The level of hs-CRP does not differ in LVSD of various aetiologies.[67] hs-CRP can predict the development of heart failure in general population and in patients who suffer from acute myocardial infarction.[67] It is also an independent prognostic marker in patients with chronic and acute decompensated heart failure.[67] Some studies have demonstrated that the prognostic value of hs-CRP in patients with CHF is incremental to that of BNP.[68,69,174,175] With concomitant measurement of NT-proBNP and TnI, hs-CRP is also useful in multi-marker approach for risk stratification in patients with CHF and LVSD.[176] However, the prognostic value of hs-CRP in patients with heart failure and PSEF remains unclear.[177]

1.6 Heart-type fatty acid-binding protein (H-FABP)

H-FABP belongs to a family of protein called the fatty acid-binding proteins (FABPs).[170,178] These FABPs are low molecular weight (15 kDa) soluble proteins that present abundantly in the cytoplasm of cells with active fatty acid metabolism such as cardiomyocytes and hepatocytes. There are nine distinct types of FABP and each has a characteristic tissue distribution and stable intracellular half-life of 2 to 3 days.[179] FABPs facilitate intracellular long-chain fatty acid transport and the delivery of fatty acyl co-enzyme A to mitochondria for oxidation and energy production.[19] They protect cells against the effects of locally high concentration of long-chain fatty acid induced by processes such as endurance exercise, ischaemia, diabetes, hypertrophy and lipid-lowering medication.[180] FABPs also regulate gene expression by mediating fatty acid signal translocation to peroxisome proliferator activated receptors.[178]

H-FABP is specific to cardiomyocytes. It is present up to 10 folds lower in skeletal muscles than cardiomyocytes and even lower in other tissues such as intestine, kidneys and brain.[181] The gene encoding for H-FABP is located on chromosome 1 and its' genetic code is FABP-3.[182,183] H-FABP is composed of 132 amino acids and have 20 – 80% amino acid sequence homology to other FABPs.[184] Therefore identification of H-FABP in other tissues using earlier polyclonal antibodies may be due to cross-

reactivity (detection limit 1 ng/ml and up to 5% cross-reactivity with other FABPs) of the assays with other fatty acid-binding proteins (FABPs).[184,185] The newer monoclonal antibody assay such as the one used in our study (HyCult Biotechnology, Uden, The Netherlands) has better sensitivity with much lower likelihood of cross-reactivity with other FABPs (detection limit 0.25 ng/ml with <0.005% cross-reactivity with other FABPs).[186]

The diagnostic potential of H-FABP in detecting myocardial injury was first discovered by Prof. Glatz in 1988.[187,188] In contrast to myofibril proteins, H-FABP is released rapidly from cardiomyocytes during myocardial injury even in the absence of myofibril damage or necrosis. In acute MI, H-FABP is detectable within 30 minutes after the onset of ischaemic episodes and rapidly increases beyond the diagnostic level within 3 hours. The level peaks at about 4 to 6 hours before returning to baseline level within 20 hours.[22] These features make H-FABP a potentially more sensitive and reliable biomarker for ongoing myocardial damage than troponins. In patients with decompensated heart failure, H-FABP is more likely to be detactable than TnT suggestive that H-FABP may be more sensitive than troponins in identifying patients with ongoing myocardial damage.[187]

H-FABP is renally excreted and so may persist longer in patients with renal impairment.[19,189] This may also partly explain the higher H-FABP level in older patients since renal function decreases with age.[190] However, it has been shown that infarct size can be accurately calculated using H-FABP and individually estimated renal clearance rate.[26-28,30,31] Therefore clinical interpretation of H-FABP level has to be taken in conjunction with renal function. In the absence of significant renal impairment, H-FABP has incremental prognostic value in addition to BNP in a selected group of patients with decompensated heart failure.[32] However, the use of H-FABP in unselected group of patients with a wider range of renal function and stable CHF has not been investigated.

1.7 Biomarkers for Haemostasis and endothelial dysfunction in CHF

Thrombus formation occurs when coagulation cascade, the extrinsic and/or intrinsic pathways, is activated. This leads to the generation of activated thrombin which cleaves fibrinogen soluble fibrin molecules (monomers) that polymerise spontaneously into the double-stranded protofibrils. These double-stranded protofibrils

can associate laterally with each other to form fibers, which in turn, can associate themselves to form fiber bundles. Collectively, the protofibrils, fibers and fiber bundles constitute the fibrin clot, insoluble gel which serves as the scaffold for thrombus formation.[191] On the other hand, thrombin is very rapidly bound and inactivated by anti-thrombin III, forming the thrombin-anti-thrombin III complex (TAT).

The fibrin mesh formed traps circulating platelets. Platelets are activated when they come into contact with thrombin, collagen and vWF. Activated platelets excrete the contents of their dense (ADP or ATP, calcium and serotonin) and alpha (α) (platelet factor 4, transforming growth factor-β1, platelet-derived growth factor, fibronectin, β-thromboglobulin, vWF, fibrinogen and coagulation factors V and XIII) granules. Platelet activation leads to initiation of arachidonic acid pathway that produce thromboxane A_2 which, together with ADP, stimulate platelet aggregation. The main receptor responsible for platelet aggregation is the abundantly present calcium-dependent glycoprotein IIbIIIa receptor. Activated platelets bind to fibrin and vWF through GPIIbIIIa receptor and to the collagen via glycoprotein 1a receptor to form platelet plug. During aggregation, the myosin and actin filaments in the platelets are stimulated to contract and reinforcing the plug.

Fibrinolysis is achieved via the action of plasmin. Its precursor, the plasminogen is produced by the liver. Plasminogen is inactive but has affinity to thrombus and is incorporated into the thrombus during thrombogenesis. Tissue plasminogen activator (t-PA) and urine-type plasminogen activator (u-PA or urokinase) convert plasminogen to plasmin which cleaves the specific bonds on the fibrins and fibrin clots producing polymers of different molecular weight and solubility. Larger polymers are further cleaved into smaller ones, forming different soluble fibrin degradation products including the D-dimer.[191] Plasmin can stimulate further plasmin generation by inducing the production of t-PA and u-PA. In contrast, α_2-antiplasmin (covalently bound to polymerising firbin by activated FXIII) and α_2-macroglobulin inactivate plasmin. Plasmin activity is also reduced by thrombin-activatable fibrinolysis inhibitor (TAFI) that makes fibrin more resistant to tPA-mediated activity. In addition, t-PA and u-PA activities can be inhibited by plasminogen activator inhibitor-1 and 2 (PAI-1 and 2).

Endothelium is important in the maintenance of normal haemostasis. Indeed, it has many other diverse physiological roles including regulation of vascular tone and permeability, metabolism, inflammatory/immune response and tissue healing. It is responsible for the synthesis of and serves as the reservoir for many biologically active

molecules and its surface has many metabolically active structures. These molecules or surface structures will be synthesized, released or activated in response to a multitude of physiological and pathological stimuli. Endothelium takes part in physiological homeostasis and disease processes hence there is a continuum of endothelial activation, dysfunction and damage. Endothelial dysfunction plays a part in the syndrome of heart failure and many treatment of heart failure have been shown to improve its function.[192] Methods that help to assess endothelial (dys)function may help to guide therapy and stratify risk in patients with heart failure.[192]

1.7.1 Fibrinogen

Fibrinogen (FBG) is the 'building block' of thrombogenesis. It is a soluble plasma protein with molecular weight of 340 kDa. Each FBG molecule consists of 3 pairs of disulfide-bonded α-, β- and γ-chains. These form a central globular E region connected by coiled-coil regions to two identical globular D regions and two αC regions (consist of approximately two-thirds of the carboxyl-terminal end of the α-chain).[193,194] A pair of disulfide rings located between the E and D regions in each half of the molecule link the chains together (α to β, β to γ and γ to α).[195] There are multiple thrombin- and plasmin-sensitive bonds within the molecules.[196]

During thrombogenesis, activated thrombin acts via proteolytic removal of fibrinopeptide A and B from fibrinogen to form soluble fibrin molecules (monomers). This exposes two polymerisation sites in the E region to which one D region of each of the two adjacent fibrin molecules on the opposing strand can attach (non-covalent bonds) such that the fibrin molecules spontaneously polymerise into the double-stranded protofibrils. In the presence of calcium, Factor XIII (a thrombin-activated enzyme) then forms a polypeptide covalent bond that crosslinks two adjacent fibrin molecules within each of the strand of the protofibrils at the D-region (of the γ-chain). The protofibrils can associate laterally form fibers which in turn, can associate themselves to form fiber bundles. These constitute the fibrin clot, insoluble gel which serves as the scaffold for thrombus formation.[191]

FBG level is raised in patients with CHF compared to normal population.[197-200] The level is correlated to inflammatory markers such as CRP, therefore the production of FBG in patients with CHF may be in part, related to inflammatory response and resultant increase in hepatic synthesis.[199,201] For these reasons, FBG may also be a

marker for inflammation. The absolute and relative FBG synthesis rate is higher in cachectic chronic heart failure patients who have a greater degree of immune system activation compared to the non-cachectic patients.[201] Increase in FBG can increase plasma viscosity hence leading to abnormal rheology.[197] Therefore, fibrinogen may cause hypercoagulation in patients with CHF by increasing thrombogenesis and plasma viscosity. However, Sbarouni et al found a raised FBG in only 1 of the 21 stable chronic heart failure patients,[55] whilst Lip et al found that FBG only increased in patients with LVSD in the presence of LV aneurysm when compared to normal controls and the level was not affected by warfarin.[202] These differences can be partly explained by the difference in patient characteristics including severity of CHF and treatment. Although the plasma FBG level is not affected by aetiology of CHF[200], it (and vWF) positively correlates to NYHA[197] but negatively to LVEF.[203] FBG level reduces following introduction of ACEI[197] and after heart transplant.[198]

Although FBG correlates with non-fatal thromboembolic event and increased long-term cardiovascular death after acute myocardial infacrtion (MI), it has not been found to be of any significant prognostic value in patients with CHF.[60,204]

1.7.2 D-dimer

D-dimer (DD) is one of the fibrin degradation products (FDPs) generated from the lysis of fibrin component of thrombus by the action of plasmin. Plasmin cleaves the crosslinked fibrin mesh of thrombus into high molecular weight polymers. These polymers are further cleaved several times into smaller polymers, the FDPs. DD molecule consists of one D region of each of the two adjacent and covalently bonded fibrin monomers within the same strand of the protofibril and the non-covalently bonded E region of the fibrin monomer from the opposing strand.[191] The crosslink between the two D region remains intact and are exposed to the surface. The molecular weight of a DD is 260 kDa. DD assays depend on the binding of a monoclonal antibody to a particular epitope on the D-dimer fragment.

DD is a marker for thrombus formation and is well established for its diagnostic use in venous thromboembolic diseases. DD is raised in patients with stable[198,205] or decompensated[56] heart failure and in both patients with heart failure and LVSD[56,57,205] or PSEF.[57,206] The degree of raised DD is higher in LVSD compared to PSEF[206]. Compared to normal volunteers, DD is higher in patients with CHF either due to

ischaemic heart disease[55,207], dilated cardiomyopathy[208,209] or hypertrophic cardiomyopathy[209]. The level is not affected by the aetiology of LVSD[55] or the presence of LV thrombus.[208] However, Lip et al found that DD only increased in patients with LVSD in the presence of LV aneurysm.[202] As expected, DD is lower in patients taking warfarin[198,202,210] or on low molecular weight heparin.[211] In patients with advanced heart failure, DD level is also lower following heart transplant.[198] In addition to venous thromboembolism, DD is also raised in other cardiovascular condition including atrial fibrillation[212] and mitral stenosis[213].

The prognostic value of DD in unselected patients with CHF and LVSD is unclear. In 195 CHF patients without significant renal dysfunction (creatinine < 250 μg/L) and who are not on anticoagulation therapy, Jug et al found that although DD level above their cohort median of 674 μg/L was associated with increased heart failure-related hospitalisation and death, it is not an independent predictor of medium-term prognosis (median follow-up 693 days).[205] In their study, raised t-PA and PAI-1 levels were independent predictors after adjustment for NT-proBNP. In 458 patients older than 65 years with signs and symptoms compatible with heart failure attending their primary care service, Alehagen et al showed that DD above 0.25 mg/L was independently associated with cardiovascular and all-cause mortality after a median follow-up of 5.5 years.[57] This was independent of known prognostic factors such as age, BNP, NYHA and LVEF. None of these patients were taking warfarin and the prognostic value of DD was unchanged after they had excluded patients with AF, malignancy and renal impairment (creatinine > 200 μmol/L). However, 214 patients in this study were reported to have normal systolic and diastolic function on echocardiographic examination suggestive that their findings were likely to be confounded by other disease processes.

On the other hand, Marcucci et al has shown that in patients hospitalised due to decompensated heart failure, DD $\geq$ 450 ng/ml (0.45 mg/L) was independently associated with mortality after a median follow-up of 8.5 months.[56] Their finding was adjusted for factors including age, cardiovascular risk factors, LVSD, renal function, NYHA functional classification, haemoglobin, sodium, TAT, CRP, IL-6 and NT-proBNP. Patients with AF, previous thromboembolism and those who were taking warfarin had been excluded from their study.

1.7.3 Tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1)

The main plasma fibrinolytic components are plasminogen, t-PA and u-PA; whilst the main inhibitors of fibrinolysis are PAI-1 (and PAI-2 that is secreted by placenta and hence only present at detectable level during pregnancy), α_2-antiplasmin and α_2-macroglobulin. Plasminogen, t-PA and fibrin form a ternary complex during fibrinolysis, a process that is limited to the surface of the fibrin clot and does not become systemic. Activation of the fibrin-bound plasminogen by t-PA is increased by fibrin polymerization. As fibrinolysis progresses, more plasminogen binding sites are exposed leading to the formation of more plasmin. Although PAI-1 also binds to fibrin and inhibits the action of t-PA, its activity is reduced by 80 – 90% since thrombin-cleaved fibrinogen and fibrin reduce PAI-1 activity. However, as fibrin polymerises and assumes more complex structure, the accessibility for the binding of t-PA is reduced. Fully formed clots contain highly polymerised fibrin with activated FXIII-mediated cross-link making them more resistant to t-PA activity.

Both t-PA and PAI-1 have been extensively investigated for their role in the development of coronary artery disease, atheroembolism and acute coronary syndrome (ACS).[214-219]

There are few but conflicting data on fibrinolysis in patients with LVSD and raised D-dimer has been used as an indirect measurement of increased fibrinolysis.[207] However, by measuring plasmin-plasmin inhibitor complex, Yamamoto et al. showed that fibrinolytic activity was similar in patients with idiopathic and hypertrophic cardiomyopathy when compared to normal subjects in the context of increased thrombogenesis as evident by an increased in fibrinopeptide A, thrombin-antithrombin III complex and D-dimer.[209] Further, inflammation, a potent stimulator of coagulation is likely to play a part in hypercoagulation in LVSD and Interleukin-6 has been shown to activate coagulation cascade without affecting fibrinolytic system.[54]

1.7.3.1 Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a 48 kDa linear glycoprotein composed of 379 amino acids. It binds rapidly to t-PA and u-PA forming stable complex with a ratio of 1:1.[220] This is cleared from the circulation by hepatocytes. When stimulated by thrombin, activated PAI-1 is released on the surface of platelets and endothelial cells to prevent clot lysis. Activated

PAI-1 is an unstable protein with a half-life of 30 minutes but can be stabilized by vitronectin (VTN).[214] Vitronectin (encoded by VTN gene) is a glycoprotein presents abundantly in the plasma, platelet and extracellular matrix and serves to regulate proteolysis initiated by plasmin.[221,222]

The PAI-1 gene is located on chromosome 7 and several genetic polymorphisms have been described.[214] Subjects with 4G allele homozygous (4G/4G genotype) has plasma PAI-1 concentration that are 25% higher than those with 5G allele homozygous (5G/5G genotype). Though not a consistent finding, some studies have shown that the 4G/4G genotype is associated with type II diabetes mellitus (DM II), cardiovascular disease and higher risk of MI.[214]

The PAI-1 gene expression is affected by multiple factors.[214] Both PAI-1 and t-PA release is closely related to the renin-angiotensin-aldosterone system (RAAS). Angiotensin II and bradykinin stimulate the release of PAI-1 and t-PA respectively. Therefore ACEIs may inhibit the release of PAI-1 but increase bradykinin-dependent t-PA release from endothelium.[223] ARBs also reduce the production of PAI-1 blocking the AT_1 receptor.[224] Glucose, insulin and proinsulin-like molecules can stimulate the release of PAI-1.[214] Control of hyperglycaemia in patients with DM II especially by using insulin reduces plasma concentration of PAI-1.[225] A very-low-density lipoprotein triglyceride-sensitive site has also been identified in the promoter region of the PAI-1 gene, close to the 4G/5G allele site. Triglycerides can increase the production of PAI-1 by their action on this receptor and the effect is increased in the presence of insulin.[226,227] This suggests that PAI-1 can be related to various disease state of metabolic syndrome including diabetes mellitus II, insulin resistance and hypertriglyceridaemia. In addition, PAI-1 level is lower in post-menopausal women receiving estrogen-replacement therapy compared to those who are not and in premenopausal women compared to post-menopausal women suggestive that PAI-1 release can be affected by estrogen.[214]

In addition to ACEI and ARB, certain β-blockers such as carvedilol can reduce the level of PAI-1.[228] Thrombin-dependent inactivation of PAI-1 level can be potentiated by unfractionated heparin and, to a lesser extent, low molecular weight heparin.[229] In patients with ischaemic stroke, long-term aspirin and/or clopidogrel treatment also reduces PAI-1 level.[230] In contrast, long-term steroid treatment in heart transplant patients is associated with an increase PAI-1 level that may be related to the formation of intracardiac thrombi in these patients.[231] Amlodipine, a calcium channel

blocker can also increase PAI-1.[232] Patients with CHF taking warfarin have higher level of active PAI-1 than those who are not taking warfarin.[198] The reason and significant of this is unclear.

The role of PAI-1 in CHF and LVSD has not been extensively investigated although it is thought to be associated with hypofibrinolysis. PAI-1 level is raised in patients with heart failure due to LVSD or PSEF but its prognostic value is unknown.[205,206]

1.7.3.2 Tissue plasminogen activator (t-PA)

t-PA is a glycoprotein produced mainly by the endothelial cells. It is a serine protease comprised of one polypeptide chain with a molecular weight of approximately 71 kDa. The gene encoding t-PA expression is PLAT gene located on chromosome 8. t-PA is released from endothelial cells through the translocation of a dynamic intracellular storage pool (and some tumour cells). The rapid release of t-PA is essential since it is more effective if incorporated during, rather than after thrombus formation.[233] Therefore, fibrinolytic activity is not necessary reflected by plasma level of t-PA. In plasma and endothelial cells, 60-65% of t-PA are present in inactive form that complexes with PAI-1. Free active t-PA is difficult to measure in plasma therefore most clinical studies have measured circulating t-PA antigen which mainly represent the complex of t-PA & PAI-1. t-PA antigen level correlates well with PAI-1 activity or antigen, and similar to PAI-1, it is positively associated development of coronary artery disease and the risk of plaque rupture and myocardial infarction.[216,217,234]

Bradykinin is a potent stimulant for t-PA release from the endothelium.[223] This is achieved via B_2 receptor and is independent of nitric oxide synthase and cyclooxygenase pathway.[235] In patients with CHF, long-term ACEI treatment dramatically potentiate bradykinin-induced endogenous release of t-PA such that the local concentration of active t-PA approaches the level achieved in thrombolysis therapy for MI.[236]

Apart from ACEIs and ARB, t-PA level can be increased by β-blockers such as carvedilol or metoprolol tartrate.[228] Unfractionated heparin and, to a lesser extent, low molecular weight heparin can shorten t-PA-induced clot lysis time.[229] However, t-PA antigen level is not affected by warfarin.[198]

The level of t-PA antigen is elevated in patients with heart failure due to LVSD or with PSEF when compared to healthy controls.[206] Jug et al found that t-PA antigen level was an independent predictor of heart failure-related death and hospitalization in 195 stable CHF patients after a median follow-up period of 693 days.[205] Therefore t-PA may be a potential biomarker of prognosis for patients with CHF.

Since t-PA and PAI-1 are released from endothelial cells, they also reflect endothelial function.[237] Their levels have been found to correlate with markers for endothelial function such as vWF and cell adhesion molecules (CAMs). Further, t-PA plays a role in tissue remodeling by activating platelet-derived growth factor (PDGF) that stimulates fibroblast proliferation.[238]

1.7.4 Endothelial activation and dysfunction in CHF

In an average 70kg man, endothelium is estimated to have a mass equivalent to five normal hearts and an area equivalent to six tennis courts.[239] Endothelial dysfunction is an integral part of heart failure syndrome and closely related to inflammation, haemostasis and neurohormonal activation. Endothelial (dys)function and/or damage can be assessed using different methods and a few biomarkers such as vWF, soluble E-selectin (sE-Sel), soluble thrombomodulin, nitric oxide and endothelin are known to have such a role.[192] Some of these markers such as vWF, soluble thrombomodulin and endothelin may have prognostic value in patients with CHF.[192]

1.7.4.1 von Willebrand factor (vWF)

vWF is a 260 kDa multimeric glycoprotein and encoded by vWF gene on chromosome 12. It is synthesized by endothelial cells and megakaryocytes. Although vWF mRNA present in platelets and vWF is a constituent of the platelet α granules, it is thought that platelet-derived vWF remains bound to the platelet surface and does not contribute to the plasma pool of vWF.[192] Therefore circulating vWF is predominantly, if not all, derived from the endothelium making it a marker for endothelial activation, dysfunction and damage.

vWF has binding sites for FVIII, collagen, vitronectin, glycoprotein 1B (GP1B), GPIIbIIIa and heparin. Circulating inactive vWF is usually bound to and stabilises FVIII. During thrombus formation, vWF crosslinks activated platelets by binding to

GP1B and GPIIbIIIa to form platelet plug. Its collagen and vitronectin binding sites mediate binding to subendothelium and stabilises the platelet plugs.[240]

The vast majority of vWF is secreted via constitutive pathway and composed of small multimers and dimers. These are found on basal membranes or free in the plasma. The remainders are larger and functionally more active multimers stored in the Weibel-Palade bodies of endothelial cells and α granules of platelets. These are released in a regulated fashion in response to vascular injury.[240] Only these large multimeric vWF molecules are haemostatically active as they have higher affinity for the ligands. The multimer size can be decreased by thrombospondin-1 that reduces the disulfide bonds of vWF multimers.[241] The release of stored vWF is stimulated by thrombin, fibrin, histamine, complement C5a-9, adrenaline, vasopressin, nicotinc acid and cytokines such as IL-1 and TNF.[192]

Patients with CHF have raised vWF and this correlates to endothelin and E-Sel but not β-thromboglobulin suggestive that endothelium rather than platelet is the main source of vWF.[55,197,242] However, raised vWF has recently been found to correlate with P-selectin, a marker for platelet activation.[197] The level of vWF positively correlates to symptoms and clinical features of heart failure but positive correlation with the severity of LVSD has not been consistently found.[197,243] However, in patients with LVSD, vWF is higher in the presence of LV aneurysm compared to those without an aneurysm.[202] CHF patients with DM II have higher vWF than those without DM II and DM II is an independent predictor of a raised vWF level.[242,243] Women with CHF also have higher vWF compared to men with CHF.[197] vWF is raised to similar level in patients with decompensated HF and stable CHF and this is related to adverse clinical outcome in both clinical settings.[59,244,245]

In patient with CHF, treatment with ACEI reduces vWF level[197] but only certain β-blockers such as carvedilol has been shown to reduce vWF.[197,228] Warfarin may increase[241] or has no effect[246] on the vWF level; whilst cyclosporin[192] may increase the level of vWF. Anti-platelets (aspirin or clopidogrel) have not been found to affect the level of vWF in patients with HF and in sinus rhythm.[246] Following heart transplant, vWF was found to be lower but remains above the level of normal controls.[198]In healthy individuals, aspirin can reduce vWF level but heparin does not change the level of vWF.[247]

In general population, higher level of vWF has been shown to be associated with higher risk of developing coronary artery disease. In patients with proven coronary

artery disease or suffering from MI, raised vWF is associated with increased risk of MI, recurrent MI and death.[192] Early increase in vWF after NSTEMI is associated with short-term adverse cardiovascular events.[248] In the same study, patients who received enoxaparin or hirudin did not have a raised vWF level and had lower short-term events. Raised vWF in patients with CHF or decompensated heart failure is also associated with adverse cardiovascular events.[59,244,245] However, it is unclear if raised vWF contributes to the increased in cardiovascular events or patients with cardiovascular events had a worse underlying disease with more advanced endothelial dysfunction or damage.

1.7.4.2 Soluble E-selectin (sE-Sel)

E-selectin (E-Sel) is cell-surface-bound leukocyte adhesion molecule that is specific to endothelial cells. It is also known as CD62 antigen-like family member E (CD62E), endothelial-leukocyte adhesion molecule 1 (ELAM-1) or leukocyte-endothelial cell adhesion molecule 2 (LECAM2). It is coded by SELE gene located on chromosome 1. E-Sel is only expressed on the surface of activated endothelial cells and plays an important role in active and chronic inflammation. It reflects endothelial activation rather than damage.[192]

E-Sel binds to sialylated carbohydrates present on the surface protein of certain leukocytes including monocytes, granulocytes and T-lymphocytes. Cytokines produced by inflamed or injured tissue induce the expression of E-Sel. Circulating leukocytes bind with low affinity to these E-Sel and 'roll' along the endothelial surface. As this progress, chemokines released from local tissue activate the 'rolling' leukocytes, which are then more tightly bound to the endothelial surface and extravasate into the tissue. In experimental study, thrombin can induce IL-1 and TNF-alpha-independent E-Sel expression.[249]

Soluble form of E-Sel (sE-Sel) can be detected in healthy individuals. The level is raised in pathological conditions such as IHD, atherosclerosis, hypertension, diabetes, malignancy, haematological condition and septic shock.[192] However, it is unclear if these are actively or passively shed from the endothelium. The level of sE-Sel does not correlate to that of vWF in diseases such as IHD, hypertension and hypercholesterolaemia. In patients with coronary artery disease (CAD), raised sE-Sel predicts future cardiovascular death.[250]

Similar to vWF, sE-Sel is raised to the same degree in patients with CHF and decompensated HF.[59,245] In patients with CHF, sE-Sel was found to be raised in those with DM II but not in those without DM II.[242] However, NYHA and age but not DM II were independent predictors of a raised sE-Sel in these patients. In patients with CHF and in sinus rhythm, sE-Sel level was reduced by warfarin after 3 months of treatment but it was not affected by anti-platelets (aspirin or clopidogrel).[246] Study of healthy individual has shown that sE-Sel level is not affected by aspirin or unfractionated heparin.[247]

Relatively few studies are available for the prognostic value of sE-Sel in patients with heart failure. When combining patients with CHF and decompensated heart failure, sE-Sel was not found to correlate with plasma BNP and the same study showed that vWF but not sE-Sel was a predictor of combined cardiovascular death, non-fatal MI, stroke, thromboembolism and rehospitalisation.[59] sE-Sel may also have a role in predicting the occurrence of an ischeamic cardiovascular events in patients with DM II.[242]

1.7.5 Platelet activition and soluble P-selectin (sP-Sel) in CHF

Platelet abnormalities are well recognized in patients with CHF.[207,251] One common method of assessing platelet activity is by measuring plasma level of soluble P-selectin (sP-Sel) modulates interaction between platelets, leukocytes and endothelium. Patients with decompensated heart failure have abnormal surface P-selectin expression[58] and raised sP-Sel level.[197]

1.7.5.1 P-selectin (P-Sel) and soluble P-selectin (sP-Sel)

P-selectin is previously known as CD62 antigen-like family member P (CD62P), granule membrane protein 140 (GMP-140) or platelet activation dependent granule external membrane protein (PADGEM). It is the largest of the selectins with molecular weight of 140 kDa and encoded by SELP gene on chromosome 1. P-Sel is a component of the membrane of the α and dense granules of platelets and of the membrane of the Weibel-Palade bodies of endothelial cells. It is expressed only on the surface of activated endothelial cells and activated platelets following various stimulations such as inflammatory cytokines, histamine, thrombin, lipopolysaccharides or oxygen radicals.

Inhibitor of NO synthase can increase the expression of P-Sel indicating that it is also regulated by nitric oxide (NO). The main ligand for P-Sel is P-selectin glycoprotein ligand-1(PSGL-1) that is present in most leukocytes. Therefore, P-Sel plays a role in inflammation process including the 'rolling' of leukocyte on endothelial surface. It is also involved in haemostasis and may have a role in atherosclerosis and cellular signaling.[252] The functions of P-Sel tend to overlap with E-Sel.

On activated platelets, P-Sel stabilises the initial GPIIb/IIIa-fibrinogen interactions allowing the formation of larger and more stable platelet aggregates. Inhibition of platelet P-Sel can achieve 95 – 100% of de-aggregation indicating that P-Sel is the main mediator for platelet aggregation.[253] P-Sel-facilitated adhesion of platelet and neutrophils to the endothelium can also lead to further endothelial activation. It also regulates production of platelet activating factor by monocyte hence enhancing its pro-coagulant activity and prime monocytes for increased phagocytosis.[252]

Soluble form of P-sel (sP-Sel) also present in plasma and most data suggest that these sP-Sel originate from platelet and reflect platelet disturbance or activation.[252] Messenger RNA/cDNA encoding for different variants of P-Sel has been identified. Some of these encode for P-Sel molecules that lack trans-membrane protein suggestive a direct release from the endothelial cells or platelets. Some of the sP-Sel may be 'shedded' passively from damaged platelets. However, it is unknown if there is any active enzymic cleavage or other mediator-induced release of surface P-Sel.

Soluble P-Sel level is raised in various acute and chronic cardiovascular disease including CAD, ACS including MI, carotid artery stenosis and ischaemic stroke. Many of these studies did not find a correlation with sE-Sel and vWF suggesting that platelet activity as the main underlying pathophysiological process.[252] The level of sP-Sel is also raised in patients with cardiovascular risk factors such as smoker, DM II, hypertension and hypercholesterolaemia though the findings are less consistent. [Blann review] Men also have higher sP-Sel than women but age has no effect on the level of sP-Sel.

Successful blood pressure control using ACEIs and/or calcium channel blockers can reduce sP-Sel in elderly hypertensive patients. Many, but not all studies have shown that statin reduces sP-Sel level in patients with stable or unstable CAD.[252] sP-Sel can be reduced as early as 1 hour following peripheral vascular angioplasty with sustained effect. Interestingly, Ishiwata et al showed that six months following percutaneous coronary angioplasty, sP-Sel level increased by 24% in those with restenosis but did not change in those without restenosis.[254] Stopping smoking is also associated with a

reduction in sP-Sel level.[252] The effects of anti-platelet and warfarin have on sP-Sel level vary between studies.[252]

Studies investigating the prognosis significance of a raised sP-Sel in patients with peripheral vascular disease and stable or unstable coronary disease has yield conflicting results. However, sP-Sel measured in citrated plasma may have a role in stratifying risk of adverse cardiovascular events.[252]

In patients with CHF and decompensated heart failure, markers of platelet activity including sP-Sel or platelet-bound P-Sel are increased regardless of the aetiology of LVSD.[58,197,255-258] The level of sP-Sel in these patients is not affected by LVEF but often related to the NYHA functional class.[197,255-257] ACEIs and β-blockers do not affect the levels of sP-Sel in patients with CHF.[197,256] Increased in platelet activity in patients with CHF is not affected by anti-platelet therapy.[58,255,258] However, combination of aspirin and clopidogrel can inhibit platelet activation in patients with CHF but not aspirin alone.[259] It may be that CHF patients have more pronounced platelet activation and platelet activation is via multiple mechanisms including inflammation and neurohormonal factors as discussed above. This may partly explain the lack of prognostic benefit of single anti-platelet therapy in patients with CHF.[45,46] Using biomarkers of platelet activations may help to guide anti-platelet therapy in patients with heart failure but this has not been investigated. Further, some biomarkers of platelet activation such as sP-Sel has not been found to be associated with the prognosis in these patients.[255]

1.8 Enhanced external counterpulsation (EECP)

Enhanced External Counterpulsation (EECP) is a safe and effective out-patient-based non-invasive treatment for CAD, even in those who are not suitable for revascularisation. It consists of ECG-gated sequential compression of lower extremities using three pairs of pneumatic cuffs applied to the calves, thighs and buttocks. These cuffs are inflated proximally in a sequential fashion during diastole and deflated simultaneously at the onset of systole. A typical course of treatment involves 35 one-hour treatment sessions over 4 to 7 weeks.

The external mechanical activity of sequential leg compression is translated into a number of beneficial haemodynamic effects on the cardiovascular system. To a large

extent, these effects are reminiscent of intra-aortic balloon pumping (IABP) leading to diastolic augmentation and systolic unloading. In contrast to intra-aortic balloon pumping, EECP has additional effect on the peripheral venous system and increases venous return.[260,261] Overall, these improve myocardial perfusion, decrease its workload and oxygen consumption as well as increase ejection fraction and cardiac output.

1.8.1 Historical background

The concept of counterpulsation originated from a combination of two important understanding in cardiovascular haemodynamics and cardiac energetics in the 1950s. They are diastolic augmentation to increase overall coronary perfusion and systolic unloading to reduce myocardial workload and oxygen consumption. By using an experimental animal model in 1953, the Kantrowitz brothers demonstrated that perfusing coronary arteries at an elevated pressure during diastole could increase coronary blood flow by 20 to 40%.[262] In 1958, Sarnoff and co-workers demonstrated that the main determinant of myocardial oxygen consumption was the pressure or tension generated by the left ventricle (tension-time index).[263] Birtwell et al. then combined these two principles in a system that decreased left ventricular wall tension during systole and increased coronary perfusion pressure during diastole by withdrawing and reintroducing blood through femoral cannulation.[264] (3) This was later termed ‘counterpulsation’ by Gorlin and formed the foundation to the development of EECP and IABP.[265]

In the early 1960’s, Dennis et al.[266], Birtwell et al.[267] and Giron et al.[268] reported that reduction in myocardial oxygen consumption and increased diastolic perfusion pressure could be achieved non-invasively by applying external pressure to the peripheral arterial system. This led to the concept of external counterpulsation. Dennis et al.[266] and Osborn et al.[269] were among the first to report works involving external counterpulsation in both animal and human. Compared to IABP, one additional haemodynamic effect of external counterpulsation was that compression of peripheral venous bed could lead to a substantial increase in venous return.

The earlier generation of counterpulsation device was a hydraulic system which generated uniform compression to the entire lower extremities. Soroff and Birtwell were the first to report the clinical use of this hydraulic counterpulsation device in human.[270] In the late 1960’s, staffs of the Artificial Devices Section of the National Institutes of

Health in America proposed that sequential, as opposed to uniform compression of lower extremities from distal (calves) to proximal (thigh to buttock) could significantly improve the effect of external counterpulsation on diastolic augmentation and venous return.[271] This was later confirmed by various experimental and clinical studies.[260,272,273] In seven normal subjects, Langou and Cohen demonstrated a modest increase in diastolic augmentation and 12% increase in cardiac output using sequential external counterpulsation.[272] At the same time, continuous progress and refinement of the techniques and device were made including the development of the less bulky pneumatic counterpulsation device. These formed the basis for the development of the current pneumatic sequential or 'enhanced' external counterpulsation (EECP) device by Dr. Zheng at Sun Yat Sen University in China in 1983.[273]

Early clinical experience with external counterpulsation was variable depending on the study design and clinical setting. The vast majority of them were conducted in the setting of acute myocardial infarction or cardiogenic shock with short period of external counterpulsation treatment.[274-278] In 1983, Zheng et al. demonstrated that prolonged period of sequential external counterpulsation provided long-term symptomatic relief in 97% of the 200 angina patients.[273,279] (15)

The interest in EECP was popularised by Soroff and Hui in 1989 when they brought the device developed by Dr. Zheng to America for clinical trials. A commercially available EECP system was later developed by Vasomedical Inc. This system was approved by the American Food and Drug Administration (FDA) for treatment of myocardial infarction, cardiogenic shock and stable and unstable angina in March 1995. In December 1999, the American College of Cardiology evaluated and formally endorsed EECP for the treatment of patients with CCS III or IV angina which is refractory to medical therapy; and in the opinion of their cardiologist or cardiovascular surgeon, are not readily amendable to surgical intervention. EECP was later cleared by the FDA for treatment of congestive heart failure in June 2002. The Medicare has also approved reimbursement for EECP treatment in patients with refractory angina including those with co-existing LVSD (LVEF < 40%).[279]

1.8.2 Clinical application

1.8.2.1 Technical basis

EECP consists of ECG-gated sequential compression of lower extremities. Three pairs of pneumatic cuffs are applied to the calves, thighs and buttocks. These cuffs are inflated proximally in a sequential fashion during diastole and deflated simultaneously just before the onset of systole. The inflation pressure of the cuffs generally ranges from 250 to 300mmHg in order to achieve an optimal haemodynamic effects with minimal risk of barotrauma. This computerised, automated inflation and deflation is triggered by microprocessor-interpretated electrocardiographic signals. The optimal timing of inflation and deflation can be adjusted manually in order to achieve a satisfactory diastolic augmentation (DA) and systolic unloading (SU). This is guided by the diastolic and systolic waveforms from finger plethysmography.

The degree of haemodynamic changes achieved by EECP treatment is estimated using the ratio of the relative magnitude of diastolic augmentation and systolic unloading. The optimal effectiveness ratio to obtain maximum haemodynamic effects with low risk of barotrauma ranges between 1.5 to 2.0.[280] The effectiveness ratio may improve over the course of treatment. It has been reported that the greater the degree of such improvement, the greater the reduction in angina class.[281] In addition, patients with higher effectiveness ratio (>1.5) at the end of EECP treatment tend to have greater angina reduction at 6-month follow-up.[282] Diastolic augmentation was also found to be an independent predictor of improved outcome following EECP in 3536 patients registered with International EECP Patient Registry (IEPR).[283] However, earlier reports found no association between diastolic augmentation with immediate and 6-month clinical outcome.[284] Other patient factors may be affecting diastolic augmentation and are more important predictors of outcome following EECP treatment.[285] The independent factors which predict higher diastolic augmentation at the beginning and end of EECP treatment are: male gender, younger age (<65 years), non-smoking and no history of diabetes, heart failure, noncardiac vascular disease, multivessel coronary artery disease or prior bypass surgery.[281,282]

1.8.2.2 Treatment regimen

A full course of EECP treatment consists of 35 one-hour treatment sessions. The conventional regimen is one treatment session administered on each of the five consecutive weekdays over a period of 7 weeks. This is based on the finding from

Zheng et al. that in 15 caronary artery disease patients there was a dose-dependent increase in exercise tolerance from 12 and 24 to 36 treatment sessions. Thereafter, the increase in exercise tolerance plateaus off.[273] However, for practical reason two sessions can be administered per day over a period of 3 to 4 weeks. Though effective, the clinical outcome of this modified regimen has never been compared to that of the initial regimen. On the other hand, for the patients who do not improve significantly after 35 treatment sessions, the course can be safely extended. Patients experience a recurrence of their symptom can receive repeat EECP treatment with good symptomatic relief in a majority of them.[286]

1.8.2.3 Patient selection

At present, though EECP has been approved by the American FDA for treatment of angina, unstable angina, congestive heart failure, cardiogenic shock and acute myocardial infarction, its application in clinical practice is mainly limited to chronic angina and a lesser extent, CHF.[287] EECP should be considered in those with refractory angina despite medical therapy and who, in the opinion of a cardiologist or cardiothoracic surgeon, are no longer a candidate for further revascularisation interventions.[288,289] EECP can also be offered to patients who opt against revascularisation interventions or when a delay in such procedures is anticipated. Clinical use of EECP in other cardiac conditions mentioned above remains to be further elucidated.

1.8.2.4 Precaution and contra-indications

Despite the fact that EECP is a non-invasive treatment with little major adverse effects, some precautions have to be exercised especially in certain clinical settings. EECP is contraindicated in moderate to severe aortic insufficiency where regurgitation could prevent satisfactory diastolic augmentation and retrograde diastolic aortic flow during EECP treatment may aggravate aortic regurgitation leading to increase end diastolic pressure and pulmonary congestion. However, patients with aortic or mitral stenosis have been treated successfully despite concerns that increased preload could lead to pulmonary congestion of heart failure.[287] Although EECP treatment has been

found to be safe in patients with congestive heart failure, it is contraindicated in the presence of decompensation.

The fact that EECP cuff inflation and deflation are triggered by microprocessor-interpreted ECG signals, presence of arrhythmias such as frequent ectopics, atrial flutter, atrial fibrillation and ventricular tachycardia may interfere with the triggering mechanism and causes patient discomfort. EECP is safe in patients with permanent pacemaker or implantable cardiac defibrillator. It is important that patients with rate response feature in their pacemakers should have this feature adjusted or deactivated during EECP treatment sessions to avoid unnecessary heart rate increase caused by patient movement.

Severe hypertension ($\geq$180/110mmHg) is a contraindication as EECP may cause a further increase in diastolic pressure above an acceptable limit. Hence blood pressure should be controlled before administration of EECP treatment. Severe peripheral vascular disease can compromise the effective counterpulsation due to reduced vascular volume and musculature of lower extremities. It is listed as a contraindication to EECP treatment especially if the patient has sores or rest pain. However, a report from IEPR has shown that despite lower diastolic augmentation in patients with non-cardiac vascular disease, the extent of benefit is comparable to those without non-cardiac vascular disease.[290] Nevertheless, abdominal aortic aneurysm is a contraindication as increase in diastolic and mean arterial pressures may aggravate the progression.

Patients who have an invasive cardiovascular procedure should be allowed sufficient time for wound healing before EECP treatment is initiated. It is recommended that EECP treatment should be delayed for one to two weeks after femoral arterial puncture cardiac catherisation and at least three months after open-heart surgery. EECP is contraindicated in patients with history of recent deep vein thrombosis or thrombophlebitis due to the potential risk of thromboembolism. Caution should be taken when consider EECP in patients with bleeding diasthesis or taking anti-coagulation therapy. In general, it is recommended to keep the INR below 2.0. EECP treatment is also contraindicated in pregnancy due to the potential danger to the fetus.

1.8.2.5 Adverse events

The non-invasiveness of EECP makes it a safe treatment with relatively few adverse effects. The most common device-related adverse effects are skin irritations

(bruise, abrasion or blister) and leg or back pain. Other reported minor adverse effects are swelling or paraesthesia of the legs. However, these rarely lead to withdrawal from treatment.[291-293] The overall severe clinical event rate during EECP treatment period is low. The IEPR registry reported that only 1.1% of patients withdrew from treatment due to a major cardiac event including death, myocardial infarction and revascularisation by conventional means (CABG or PCI). There was 2.4% of the patients experienced unstable angina and 2.1% developed decompensated heart failure.[293]

In general, EECP is safe across a wide range of patient type and age span. The major adverse events are not significantly greater in subgroup of patients who are, conventionally, known to be at higher cardiovascular risk such as elderly[294], diabetics[295] or patients with significant left main coronary artery disease[296], CHF[297] and aortic stenosis.[298] However, patients with history of CHF are more likely to have exacerbation of heart failure during treatment period at a rate of 5.5% compared to 0.2% in those without CHF.[299] However, the composite major adverse cardiovascular events (MACE) including death, myocardial infarction, percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery (CABG) occur at the similar rate in those with and without a history of CHF.

1.8.3 Haemodynamic effects

During EECP treatment, the external mechanical activity of sequential leg compression is translated into a number of beneficial haemodynamic effects on the cardiovascular system. To a large extent, these effects are reminiscent of intra-aortic balloon pumping (IABP) leading to diastolic augmentation and systolic unloading. In contrast to intra-aortic balloon pumping, EECP has additional effect on the peripheral venous system and increases venous return.[260,261] The acute haemodynamic effects during EECP has been well studied using various invasive and non-invasive methodologies such as finger plethysmography[281,282,284], thoracic electrical bioimpedence[300], duplexsonography or Doppler echocardiography[280,301] and invasive cardiovascular catheterisation.[280,302] However, the longer-term effects have not been well characterized.

During diastole, the sequential distal-to-proximal compression of lower limb arterial vasculature induces retrograde flow of blood from lower limbs to central aorta. With effective diastolic augmentation, Doppler echocardiographic measurement of

retrograde flow in abdominal aorta can increase by 135% during EECP.[261] The extent of diastolic augmentation achieved during EECP is similar to that achieved by IABP.[301] The significant increase in systemic diastolic pressure enhances perfusion to various organs.[301] (Werner AJC;1999) Of particular importance is the coronary vasculature which receives majority of its blood supply during diastole. In patients with coronary artery disease, EECP increased diastolic flow volume by 42±2% in left coronary main stem.[302] EECP also increased intracoronary diastolic pressure by 28% and peak Doppler flow velocity by 150%.[302] These increases have a linear relationship to the inflation pressure of the pneumatic cuffs. On the other hand, aortic pressure decreases during systole leading to systolic unloading.[302]

EECP also increases venous return through the compression on the lower limb venous system. Elegant invasive cardiac catheterisation studies by Taguchi et al.[261] and Michaels et al.[303] have both shown an increase in right atrial pressure during EECP treatment consistent with increased venous return. Taguchi et al. found that RAP and pulmonary capillary wegde pressure (PCWP) increased after 30 minutes of EECP treatment but this returned to baseline level 45 minutes into a treatment session, an effect that was not seen in patients receiving IABP.[261] The normalisation of RAP and PCWP coincided with the increase in cardiac index (CI). On the other hand, Michaels et al. observed an increase in LV end-diastolic and end-systolic volumes (LVEDV and LVESV) consistent with increased LV filling.[303]

Arora et al. studied the acute and chronic haemodynamic effects of EECP using thoracic electrical impedence measurement.[300] After 1 hour of EECP, LVEDV index decreased with associated reduction in stroke volume (SV) and cardiac output (CO) but an increase in the systemic vascular resistance.[300] However, after 35 treatment sessions, only SV remained significantly reduced with an associated increase in the index of contractility and thoracic fluid index. Urano at al. has shown that a course of EECP improved LV diastolic filling and reduced LV end-diastolic pressure (LVEDP) in patients with stable CAD.[304] Arora's study involved patients with chronic stable angina, whilst Michaels' involved patients with normal LV function referred for cardiac catheterisation and Taguchi's involved patients with acute MI.

In 47 patients with LVSD due to CAD, Kozdag et al. has shown that LVEF increased following a course of EECP treatment.[305] EECP can also improve left ventricular function independent of changes in haemodynamics. A course of EECP was found to be associated with significance increase in LV preload-adjust maximal power

and ejection fraction.[306] Similarly, using bioimpedence measurement, EECP increased maximum cardiac power by 32% with associated increased SV and CI in 5 patients with CAD and LVEF of 35% but not in 20 patients with LVEF > 35%.[307]

1.8.4 Mechanisms of action

The mechanism(s) of action of EECP is unclear but may be multiple. EECP potentiates the recruitment of collaterals and promotes angiogenesis.[308] Experimental canine model has shown that EECP increase myocardial capillary density in experimental acute myocardial infarction with associated improvement in myocardial perfusion on radionuclide scan.[309,310] In porcine study, a course of EECP increases arterial wall shear stress activates endothelial NO synthase/NO pathway[311] and down-regulate pro-inflammatory cytokines.[312] These inhibit hypercholesterolaemia-induced intimal hyperplasia and development of atherosclerosis by reducing endothelial damage, stopping vascular smooth cell proliferation and migration, and suppressing extracelluer matrix formation.[311] A course of EECP also increase the expression of granulocyte colony-stimulating factor (G-CSF), mobilises endothelial progenitor cells and increases in regional myocardial angiogenesis in hypercholesterolaemic porcine model.[313] In clinical studies of EECP, shear stress on vascular endothelium induced by EECP up regulates the expression of various angiogenic growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatic growth factor (HGF).[314]

EECP improves endothelial function and modulate endothelial nitric oxide (NO) and endothelin-1 (ET-1) release. EECP causes dose-dependent increase in NO and decrease ET-1 which can be maintained for up to 3 months after treatment.[315,316] In addition, EECP improves peripheral macro- and/or microvascular endothelial function in patients with symptomatic CAD or patients with LVSD due to CAD.[317-320] It is known that peripheral endothelial function correlates closely to coronary endothelial function.[321] Coronary endothelial dysfunction is associated with myocardial perfusion abnormality. The improvement in endothelial function has been shown to be associated with an improvement in the doppler assessment of coronary diastolic peak flow velocity and coronary flow reserve in patients known to have coronary slow flow.[322] This improvement was found to have an inverse relationship with the change in hs-CRP

level, suggestive that the effect may be, in part, achieved through modulation of inflammatory pathway or cytokines.[319,322]

Clinically, EECP improves myocardial perfusion on radionuclide and PET imaging.[304,323-327] In a multicentre observational study that enrolled 175 patients, Stys et al. showed that EECP treatment improved the perfusion defects seen on exercise-treadmill stress radionuclide scan in 54 – 85% of the patients.[324] In 12 patients with stable CAD, Urano et al. has shown that a course of EECP treatment was associated with an improved exercise tolerance and a reduction in the prevalence of exercise-induced reversible perfusion abnormality on thallium radionuclide imaging.[304] Using ^{13}N-ammonia PET scan, Masuda et al. showed that EECP improved myocardial perfusion and coronary flow reserve at rest and with dipyridamole.[323] In addition, EECP also reduces wall motion abnormality during dobutamine-stress echocardiography[328] and this may be related to the severity of coronary disease or the presence of collaterals.[329] However, smaller multi-centre study using technetium Tc 99m sestamibi radionuclide scan[330] and single-centre study using ^{13}N-ammonia PET scan[331] did not show any improvement in myocardial perfusion despite an increase in exercise capacity following EECP treatment. It is plausible that only certain patients would benefit from EECP.

EECP also favourably modulates the renin-angiotensin system (RAS). RAS plays an important pathophysiological role in LVSD and CAD and has been the strategic target for heart failure treatment.[332] A course of EECP is associated with significant reduction in plasma renin, angiotensin converting enzyme and angiotensin II levels.[333] As mentioned earlier, EECP can also improve left ventricular function independent of changes in haemodynamic leading to an increase in load-dependent LV maximal power and ejection fraction.[306]

However, some evidence has suggested EECP may also exert a peripheral effect similar to that of exercise training.[325,334] This is not surprising as EECP may theoretically cause passive mechanical stimulation of lower limb muscles and brings about various benefits similar to the effects of exercise training.[335,336] Only a small increase in peak oxygen uptake ($pVO_{2)}$ occurs during a session of EECP can be observed.[334] This increase is equivalent to a very low level of exertion and, although unlikely to induce a significant training effect, the minimal effective exercise intensity for increasing cardiorespiratory fitness in unfit or fit patients with and without CAD is lower than previously observed, 30% - 45% of the VO_2 reserve.[337]

In the presence of limited randomised controlled trial data and the fact that appropriate control is not easy to be established in device therapy such as EECP[338], the possibility of placebo effect and its extent could not be excluded or defined.[339]

1.8.5 Clinical experience

As mentioned in the historical background, earlier clinical experience in EECP was mainly in conditions such as acute myocardial infarction and cardiogenic shock before its use in chronic angina and CHF was explored.

Most of the clinical experience in EECP has been based on observational data. The Multicenter Study of Enhanced External Counterpulsation (MUST-EECP) randomised 139 patients with chronic angina in 7 centres to a full course of active EECP versus sham-placebo control with lower cuff inflation pressure of 75 mmHg.[291] Patients who received the active treatment experience an improvement in exercise time and time to >= 1-mm ST-segment depression during exercise stress test, although the controls had similar extent of increase in exercise time. However, more patients in the active group experience an improvement in their angina control when compared to the controls. The improvement in quality of life in the active group was sustained for at least 1 year.[340]

A vast 'real world' clinical experience in treating patients with refractory angina has been gathered from the International EECP Patient Registry (IEPR).[341] IEPR Phase-1 enrolled 5000 patients with refractory angina and the intended follow-up period was 3 years. IEPR Phase-2 enrolled a further 2500 patients with refractory angina or CHF and additional Kansas City Cardiomyopathy Questionnaire data were collected from the patients. Consecutive patients treated with at least 1 hour of EECP in participating centres were enrolled in the registry and therefore the data reflect actual clinical setting. In general, EECP is safe and effective in improving angina control. Approximate 75% of the patients can be expected to gain at least an improvement of angina by 1 CCS angina class with reduction in angina frequency and short-acting nitroglycerin (GTN) use following a course of EECP treatment and the beneficial effects can be sustain for up to 2 years in the survivors.[342] However, a small study has suggested that the beneficial effects may last for up to 5 years in some patients.[343]

Overall, men, non-smoker, more severe angina and absence of history of CHF, diabetes and CABG are predictors of favourable immediate response to EECP[344] whilst

initial positive response to EECP, better baseline CCS class and absence of history of CHF are predictors of sustained angina improvement without an MACE for at least 1 year.[345] EECP treatment can also be repeated safely with over 65% of the patients can be expected to experience an improvement in their angina control.[286] As mentioned earlier, many observational studies have shown that the improvement in angina control is associated with the improvement in myocardial perfusion based on radionuclide imaging or dobutamine stress echocardiogram.

Much clinical experience of EECP in patients with CHF can be learnt from the IEPR. Compared to those without a history of CHF, patients with CHF were more likely to experience an exacerbation of CHF during treatment period (0.2% vs 5.5%, $p<0.001$), and smaller proportion of them experienced an improvement in angina control (75.1% vs 68.3%, $p<0.01$).[299] Similar degree of benefit can be gained by those with LVSD or preserved systolic function.[346] Patients with angina and LVEF < 35% will also experience sustained improvement in angina following EECP therapy for at least 2 years.[347]

The Multicenter Feasibility Study treated 26 patients with stable CHF and NYHA II-III with a standard course of EECP treatment and none of them experience an exacerbation of CHF during treatment period with negligible cardiovascular events.[348] These patients had sustained improvement in exercise tolerance, peak oxygen uptake and quality of life for at least 6 months. The prospective Evaluation of Enhanced External Counterpulsation in Congestive Heart Failure Study (PEECH) randomised 187 patients with LVEF <= 35% to optimal medical therapy versus optimal medical therapy and EECP showed a benefit, especially in patients older than 65 years of age, in exercise tolerance, symptom and quality of life but the effect on LV function and laboratory blood tests including natriuretic peptides was not reported.[292,297,349] Echocardiographic study on 47 patients with LVSD and CAD suggests a potential benefit in LV systolic function improvement and associated reduction in NT-proBNP following a course of EECP treatment.[305] The past experience of EECP in patients with CHF will be elucidated further in Chapter 6.

As EECP has a systemic effect and improves perfusion to all organs during the treatment,[301] its potential uses in other clinical conditions have also been studied by many in the recent years. These include coronary vasospasm[350], takotsubo[351], peripheral vascular disease[352], retinal artery occlusion and ocular ischaemic diseases[353,354], erectile dysfunction[355], renal function[356,357], cognitive function[358] and experimental animal

model to improve cerebral perfusion following resuscitation for cardiopulmonary arrest.[359]

1.9 Conclusion

The interactions among haemostasis, inflammation, neurohormonal activation, on-going myocardial damage and endothelial dysfunction in patients with CHF due to LVSD are not fully understood. BNP and to a much lesser extent, markers for inflammation and ongoing myocardial damage have prognostic value in patients with CHF. The prognostic value of biomarkers for haemostasis and endothelial dysfunction remains questionable. Further, previous studies investigating the prognostic value of multiple biomarkers in patients with LVSD were small and/or consisted of selected group of patients and most focused on few aspects of these interactions.

By focusing on different aspects of heart failure syndrome, the studies in this thesis aimed to investigate the potential prognostic value of H-FABP (marker of myocardial injury or ongoing myocardial damage), D-dimer and fibrinogen (markers of thrombosis), tissue plasminogen activator and plasminogen activator-1 activity (markers for fibrinolytic activity), von Willebrand factor activity (vWF) and soluble E-Selectin (E-Sel) (markers for endothelial function) and soluble P-Selectin (P-Sel) (marker for platelet activation) in unselected patients with CHF on stable medication due to LVSD. The change in levels of these markers with time was evaluated for their potential value in dynamic risk stratification. Whether any of these markers have incremental prognostic value in addition to NT-proBNP and/or hs-CRP and whether multi-marker assessment would be a better risk stratification strategy was also explored.

In addition, patients with CHF have impaired quality of life and exercise tolerance despite modern treatment regimen, the potential roles of EECP to improve these aspects of CHF treatment, and its effects on LV function and some laboratory markers were also investigated.

Chapter 2 Methodology

2.1 Introduction

This chapter gives an overview of the design, patients, methods and some specific statistic considerations of the thesis. The details will be outlined in each relevant chapter.

2.2 Design

The thesis is made up of a few studies which can broadly be divided into 2 parts. The first part is based on observational prospective studies. These studies are divided into four sections:

1) Cross-sectional study to investigate the values of H-FABP and haemostatic markers in stratifying the risk of patients with stable CHF. This is the main interest of the thesis.

2) Longitudinal study to investigate the effect of heart failure treatment or treatment optimisation has on H-FABP and haemostatic markers. The prognostic value of the change in these biomarkers will also be investigated.

3) Longitudinal study to investigate the change in the level of these biomarkers with time and whether these changes would help in dynamic risk stratification of patients with stable CHF.

4) Longitudinal study to investigate the usefulness of red and white cell variables derived from routine full blood count (FBC) as independent prognostic marker.

The second part of the study investigated the potential use of EECP in patients with left ventricular systolic dysfunction due to ischaemic heart disease (IHD). This part is also divided into two sections:

1) Observational study based on the data from the International EECP Patient Registry (IEPR) to investigate the safety and efficacy of EECP in improving the quality of life in patients with angina and CHF.
2) Randomised study of EECP in patients with LVSD and IHD.

2.2.1 Patients and study subjects

Consecutive patients with LVEF $\leq$ 40% attending the Heart Care Clinics at Castle Hill Hospital and Hull Royal Infirmary were approached and those who gave written consent were included in the studies for the first part of this thesis. The distribution of patients was:

1) 500 patients with stable CHF were planned to be included in the cross-sectional study.

2) Another group of 100 new patients who were referred to the Heart Care Clinic for the diagnosis and/or management of CHF due to LVSD were planned to be enrolled. When their medication regimen and CHF were considered to be at a stable state after 4 to 8 months, they had further blood test in order to study the effect of treatment on the biomarkers being investigated. At this stage, these patients were also included in the cross-sectional study cohort.

3) From the cross-sectional study patients, further blood samples were taken after 8 to 14 months from the patients who returned for follow-up clinic visit and consented to have further blood sample taken for the purpose of clinical research. A total of 200 patients were intended to be included for this part of analysis.

For comparison, 150 patients who were referred to the Heart Care Clinic due to suspected heart failure and in whom LVSD had been excluded following assessment were recruited. The results from the study groups were compared to a group of age- and sex-match patients from these non-LVSD patients.

In addition, 50 healthy volunteers were also planned to be recruited for comparison as well. These were departmental or university staffs and/or their spouses or relatives/friends as well as general public who were aware of our programme.

As FBC is a routine blood test performed in the first visit to the heart failure clinic for all patients, consecutive patients enrolled in the heart failure clinic with LVSD and had blood test results available including FBC, NT-proBNP and biochemical profiles were included in the analysis. The plan was to include at least 1500 patients enrolled in the heart failure clinic and gave consent to take part in research projects.

Two cohorts of patients were included for the EECP studies:

1. Consecutive patients with angina and CHF who received EECP treatment for angina and were enrolled in the Phase 2 of IEPR.
2. Patients who had LVSD and IHD with LVEF < 50% based on cardiac magnetic resonance imaging were randomised to receiving the full 1-hour session of EECP (Active) in order to compare to the brief 5-minute session as controls

2.2.2 Clinical history, examination and investigations

A detailed clinical history including medications and symptoms was recorded at every visit. All patients were assessed by a physician and had an electrocardiogram and echocardiogram. The aetiology of LVSD was ascertained by the assessing physician. Patients were classified as having LVSD due to ischaemic heart disease (IHD) if they had angiographically documented coronary artery disease or a prior acute coronary syndrome with diagnostic changes in biomarkers and electrocardiogram.

In the biomarkers studies, LVEF was calculated using Modified Simpson's Method when possible and by visual estimate when not. The degree of LVSD was classified as mild-to-moderate, moderate and severe, corresponding to LVEF 35% - 40%, 26 – 35% and < 25% respectively.

Additional tests including bioimpedance body composition test, cardiopulmonary exercise testing and cardiac magnetic resonance imaging in the EECP part of the study. Details are described in Chapter 6.2.2.

2.2.3 Laboratory investigations for the biomarker study

Biochemical profile, albumin and FBC were measured in the local hospital laboratory. Additional blood samples were collected into 3.2% sodium citrate (Greiner Bio-One GmbH, Austria) and 10.8 mg EDTA (Belliver, UK). These samples were centrifuged immediately at 3000 rpm for 15 minutes at 4 °C and the plasma was stored at -80 °C. The plasma samples were sent in batches to the core laboratory in McMaster University, Hamilton, Ontario, Canada. NT-proBNP was assayed in EDTA plasma (Elecsys 1010 analyser, Roche Diagnostics, Mannheim, Germany) whilst H-FABP was assayed in the citrated plasma (HyCult Biotechnology, Uden, The Netherlands). Anaemia was defined according to WHO classification (haemoglobin <13.0g/dL in men

and <12.0g/dL in women).[360] Creatinine clearance was estimated using the Cockcroft-Gault equation.[361]

2.3 Statistical consideration

Continuous variables are presented as mean ± standard deviation (SD) if normally distributed and as median (inter-quartile range) if not. Categorical variables are presented as percentages. All continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. For two-sample comparison of continuous variables, two-tailed unpaired *t* test was used for normally distributed variables and Mann-Whitney U tests otherwise. For paired sample comparisons, paired t test was used for small sample size and normally distributed data; whilst Wilcoxon test for if data were skew. The Chi square test was use for between-group comparisons of categorical variables except when the expected values in any of the cells of the contingency table were below 5, in which case Fisher's exact test was used. McNemar's test was used for paired sample categorical data comparisons.

Where uni-variable and multi-variable binary logistic regression analyses were performed, the results were presented as odd ratios (OR) and 95% confidence intervals (CI) are presented. The Cox model is semiparametric in that no assumption concerning event-free survival time is necessary. The Cox regression model is based on the assumption that the effect of a risk factor is constant over time. The assumption of proportionality was tested by residual plotting.[362,363]

Correlations between continuous variables were assessed using Pearson correlation if normally distributed and Spearman's rho if otherwise. Receiver Operative Characteristic (ROC) curve analysis was used to compare the area under the curve (AUC) of different biomarkers using the methods described by Cleves[364] and to determine the cut-off threshold (Youden points) for some of the biomarkers.

Kaplan Meier Curves were used as the unadjusted method to assess the prognostic value of some of the biomarkers based on the threshold or classification established from the studies. The adjusted prognostic value of each of the biomarkers were assessed using uni-variable and multi-variable Cox regression analysis and the results were presented as hazard ratios (OR) and 95% confidence intervals (CI). The

proportional hazards assumption was checked for all the variables based on the plots of Schoenfeld residuals.

The incremental value of a particular biomarkers in predicting a prespecified outcome was assessed by multiple methods.[365] To estimate the prediction accuracy a biomarker, the c-statistic (or AUC) of a multi-variable logistic model that consisted of all relevant variables except the biomarker of interest (the base model) was calculated. This was compared to the c-statistic of the model with the addition of the biomarker of interest.[366] The performance of each model was evaluated using calibration. Calibration is related to the goodness-of-fit of a logistic model which is assessed using Hosmer-Lemeshow test that compares the predicted and observed outcome. The integrated discrimination improvement (IDI) was used for evaluating the improvement of model performance.[367] The net reclassification improvement (NRI) was calculated to evaluate the added predictive ability of the biomarker of interest.[367] For this analysis, 4 clinically relevant groups were derived based on the average population mortality rate of approximately 1.8% in the United Kingdom for those between the age of 65 to 74 years in 2008[368] and annual mortality rate of approximately 10% for patients with stable heart failure.[369] For example, the 4 groups of patients with the probabilities of suffering from a combined 5-year death and heart failure hospitalisation were 1) < 10% (Background-risk), 2) 10 to < 20% (Low-risk), 3) 20 to 60% (Intermediate-risk) and 4) > 60% (High-risk). The patients were reclassified according to these risk groups for probabilities of a primary event at 5 years after the addition of the biomarker of interest to the base model. The NRI is the sum of the net proportion of patients appropriately reclassified to a higher risk group in those who had an event and the net proportion of patients appropriately reclassified to a lower risk group in those who did not have an event.

The randomised EECP study involving patients with IHD and LVSD was based on the primary outcome measure of a 5% point increase in LVEF and a SD of 5%, 22 patients were required in each group in order to provide a 90% statistical power (5% significance, two-tailed). The study planned to recruit 60 patients in order to allow for a 30% dropout rate.

For all the analyses in this thesis, a nominal level of 5% statistical significance (two-tailed) was assumed throughout. All analyses were performed with a personal computer using the Statistical Package for Social Sciences (SPSS) 13.0 (IBM SPSS, Chicago, USA) and Stata 11 (StataCorp LP, Texas, USA).

2.4 Summary

The background, design and methodology of each part and section of the studies mentioned above will be discussed in more detailed in their corresponding section in the thesis.

Chapter 3 Perturbed haemostasis in chronic heart failure

3.1 Markers of disturbed haemostasis, endothelial dysfunction, inflammation and neurohormonal activation in patients with chronic heart failure and left ventricular systolic dysfunction.

Some of the preliminary short-term follow-up data of this chapter have been presented in the European Society of Cardiology Annual Scientific Congress.[370,371]

3.1.1 Introduction

Heart failure (HF) due to left ventricular systolic dysfunction (LVSD) is a systemic syndrome that is associated with disturbed haemostasis and haemorheology, endothelial dysfunction, up-regulation of the inflammatory response and neurohormonal activation. Collectively, these processes play a role in the progression of the heart failure syndrome and may be related to morbidity and mortality.

As mentioned erlier in Chapter 1.2.2, LVSD is associated with a hypercoagulable state due to the classic Virchow's Triad.[32] It remains uncertain whether anti-thrombotic therapy improve outcome in patients with chronic heart failure.[33,34,46,48,51,192]

The interactions among haemostasis, endothelial function, inflammation and neurohormonal activation in patients with left ventricular systolic dysfunction are not fully understood. Biomarkers such as N-terminal pro-B-type natriuretic peptide (NT-proBNP) and high-sensitivity c-reactive protein (hs-CRP) are also strong predictors of morbidity and mortality in heart failure.[68,69] The prognostic value of haemostatic biomarkers are less clear although D-dimer, a maker of thrombus formation, has been reported to carry incremental prognostic value over NT-proBNP or CRP in patients with heart failure.[56,57] Previous studies investigating the interaction and prognostic value of these markers in patients with LVSD were small and/or consisted of selected group of patients with acute decompensated heart failure or a mixture of systolic and diastolic dysfunction and most focused on few aspects of these interactions. [56-60]

We have investigated the effects of heart failure on various biomarkers of haemostasis, endothelial function, inflammation and neurohormonal activation and their

prognostic value in patients with at least mild-to-moderate LVSD and who were on stable heart failure treatment.

3.1.2 Methods

3.1.2.1 Patients

Four hundred and seventy three consecutive patients with LVEF $\leq$ 40% attending a local hospital heart failure service and on stable heart failure treatment were included in this study. Their laboratory results were compared to 88 age- and sex-matched patients who had or were at risk of developing cardiovascular disease and in whom LVSD had been excluded after being assessed in the same service. The service was based at Hull Royal Infirmary and Castle Hill Hospital, Kingston-upon-Hull, UK. The study was approved by Hull and East Riding Local Research Ethics Committee and the Research Board of Hull and East Yorkshire Hospitals NHS Trust. All the patients gave written informed consent.

3.1.2.2 Investigations

Detailed information on medical history including medications and symptoms were recorded at baseline. All patients were assessed by a physician and had an electrocardiogram and echocardiogram. LVEF was calculated using Modified Simpson's Method when possible and by visual estimate when not.

Blood was taken and biochemical profile, albumin, full blood count and high sensitive c-reactive protein (hs-CRP) were measured in our local hospital laboratory. Additional blood samples were collected into 3.2% sodium citrated (Greiner Bio-One GmbH, Austria) and 10.8 mg EDTA vacutainers (Belliver, UK). These samples were centrifuged immediately at 3000 rpm for 15 minutes in a refrigerated centrifuge at 4 °C and the plasma was stored at -80 °C. These plasma samples were sent in batches to the core laboratory in University of McMaster, Hamilton, Ontario, Canada. The EDTA plasma was used in NT-proBNP assay (Elecsys 1010 analyser, Roche Diagnostics, Mannheim, Germany) whilst the citrated plasma was used for the assays of D-dimer (TintElize® D-dimer, Trinity Biotech, Ireland), fibrinogen (AssayPro, Universal Biologicals, UK), vWF activity (REAADS, Corgenix, UK), t-PA and PAI-1 (HYPHEN BioMed, France), sP-Sel and sE-Sel (Bender MedSystems, Vienna, Austria). Creatinine clearance was estimated using Cockcroft-Gault equation.[361]

Patients were classified as having LVSD due to coronary artery disease (CAD) if they had angiographically documented CAD or a prior acute coronary syndrome with diagnostic changes in biomarkers and electrocardiogram. In the absence of such evidence, idiopathic dilated cardiomyopathy was recorded as the primary cause of HF if there was no documented hypertrophic or viral cardiomyopathy, significant primary valvular disease, hypertension, arrhythmia or history of excessive alcohol intake.

Patients were followed regularly in the HF management program every 4 to 6 months. Additional clinic visits were made if clinically indicated.

3.1.2.3 End point

All-cause mortality was the primary end point of the study.

3.1.2.4 Statistical analysis

Variables are presented and tested for normal distribution as described in Chapter 2.3. Two-sample comparisons of variables were also performed as described in Chapter 2.3. Multi-group comparisons of continuous variables were performed using one-way analysis of variance (ANOVA) if normally distributed and Kruskal-Wallis test if otherwise. NT-proBNP and haemostatic makers that were not normal distributed had logarithm transformation for data analysis.

Any relationship between studied variables was explored using Pearson correlation coefficient. Collinearity among variables was examined by Tolerance and Variance Inflation Factor (VIF).

Uni-variable and multi-variable Cox modelling was performed to estimate hazard ratios (HR) and 95% confidence intervals (CI) as described in Chapter 2.3.

Cox regression analyses were used to identify biomarkers that were independently associated with all-cause mortality. Haemostatic markers that predicted mortality in uni-variable analysis were analysed individually in a multivariable model that included other baseline clinical and laboratory factors that were also predictors of all-cause mortality in uni-variable analysis. Markers that remained statistically significant were included together in the final Cox regression model provided that there was no strong relationship or collinearity among them. The same variables were then used in further multivariable Cox regression model to identify if these haemostatic markers were independent predictors of cardiovascular hospitalization, cardiovascular mortality and combined cardiovascular hospitalization and all-cause mortality. For graph presentation, each of the haemostatic markers which were independent predictors

or all-cause mortality was divided into tertiles and Kaplan-Meier survival curves were plotted.

A group of patients were invited to return for a repeat blood test within 8 to 14 months following their initial assessment.

3.1.3 Results

The baseline characteristics of the patients with and without LVSD are shown in Table 3.1.1. The prevalence of ischaemic heart disease (IHD), atrial arrhythmias and renal dysfunction was higher in patients with LVSD. They were also more likely to be taking medication including angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs), aldosterone antagonists, beta-blockers, diuretics and anti-thrombotics. Those with LVSD had higher D-dimer, t-PA, sP-Sel, vWF activity, hs-CRP and NT-proBNP but lower heamoglobin, platelet count, fibrinogen and PAI-1.

In the group of patients with LVSD, NT-proBNP was related to severity of LVSD [89.0 (39.0-163.0), 123.0 (44.1-263.0) and 214.0 (97.8-416.5) pmol/L in mild-to-moderate, moderate and severe LVSD respectively, $p<0.001$]. Women had lower haemoglobin (Hb) [12.5 (11.6-13.4) v 13.5 (12.3-14.3) g/dL, $p<0.001$] and higher platelets [240 (204-282) x 10^9/L v 212 (171-248) x 10^9/L, $p<0.001$] than men. Patients with atrial arrhythmias had lower D-dimer [75.7 (34.1-241.4) v 113.0 (53.1-237.0) ng/ml, $p=0.028$] but higher NT-proBNP [202.3 (101.4-430.7) v 106.0 (44.0-238.0) pmol/L, $p<0.001$] than those who were in sinus rhythm; whilst those with co-existing non-cardiac vascular disease had higher D-dimer [156.3 (73.0-312.2) v 93.1 (42.8-209.0), $p=0.001$], NT-pro-BNP [205.0 (84.0-438.2) v 122.7 (48.0-256.0), $p=0.001$] and hs-CRP [4.6 (2.7-9.7) v 3.7 (1.6-6.9) mg/L, $p=0.026$]. The aetiology of LVSD did not affect the levels of these makers.

Diabetics had lower Hb [12.9 (11.7-13.9) v 13.4 (12.2-14.2) g/dL, $p=0.009$] and D-dimer [79.4 (34.5-159.4) v 116.1 (48.4-255.9) ng/ml, $p=0.007$] but higher PAI-1 [94.9 (64.8-150.5) v 77.0 (51.2-118.0) ng/ml, $p=0.001$] and sE-Sel [73.6 (54.6-102.4) v 59.4 (43.5-80.0) ng/ml, $p<0.001$]. Treatment with an ACEI and/or ARB was associated with lower D-dimer [94.7 (42.8-236.9) v 169.9 (93.4-237.2) ng/ml, $p=0.017$], NT-proBNP [125.9 (51.5-268.0) v 232.6 (76.0-524.0) pmol/L, $p=0.012$] and hs-CRP [3.7 (1.7-7.2) v 5.5 (3.6-15.0) mg/L, $p=0.008$] whilst β-blocker did not affect the level of

NT-proBNP or haemostatic markers. Anti-thrombotics also affected the level of D-dimer and fibrinogen. (Table 3.1.2)

The relationship between each haemostatic marker to age, GFR, log(NT-proBNP), hs-CRP, Hb and red cell distribution width (RDW) was at most, modest. (Table 3.1.3). There was no significant collinearity among these factors with the VIF of < 1.5 and tolerance between 0.84 and 0.97.

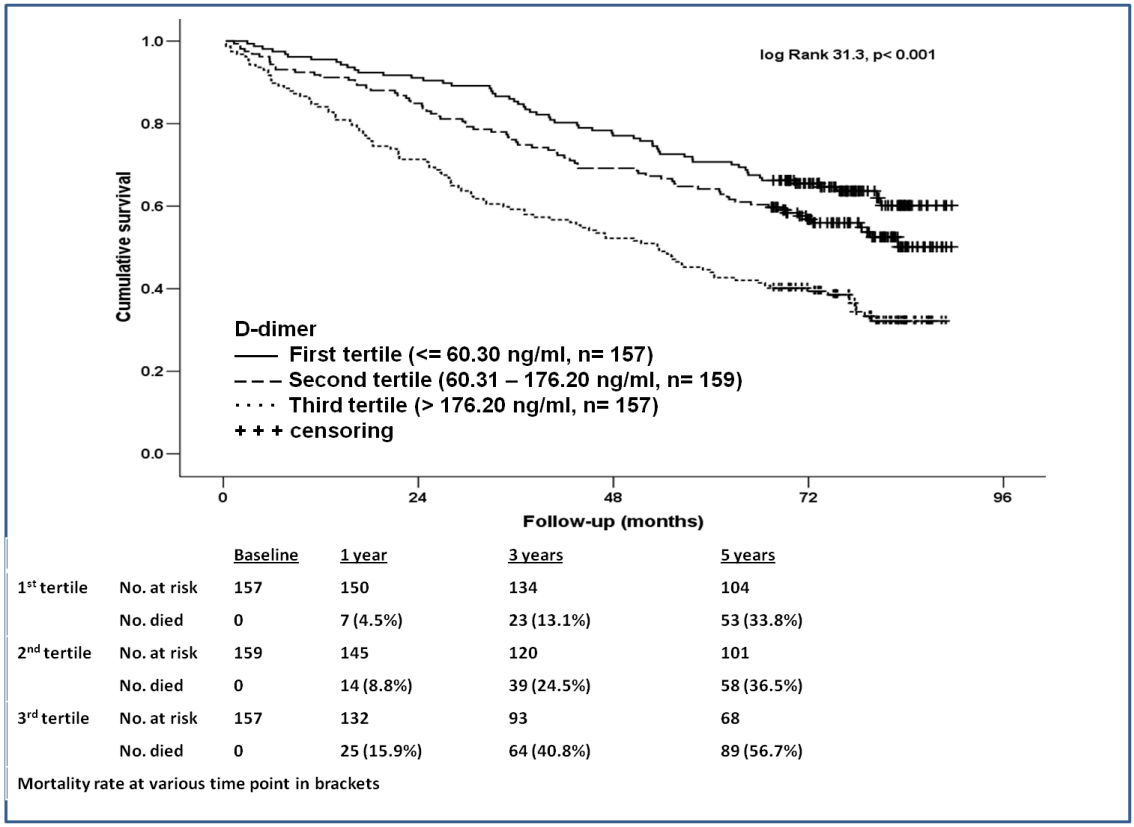
Amongst the patients with LVSD, 233 (49.3%) patients died during long-term follow-up. The survivors were followed for a mean period of 78.1 ± 6.1 (range 67.5 – 89.6) months. Of the deaths, 168 (72.1%) were due to a cardiovascular cause. Overall, 278 (58.8%) patients had at least a hospital admission due to a cardiovascular cause and 169 (60.8%) of them were hospitalised for decompensated heart failure.

By 5 years, 191 (40.4%) of the patients with LVSD had died and 142 (74.3%) of these death were due to a cardiovascular cause. Two hundred and sixty one (55.2%) patients were hospitalised for at least once due to a cardiovascular cause and 155 (59.4%) of these patients were hospitalised due to decompensated heart failure. Patients who died by 5 years were older, had higher prevalence of co-morbidities with more of them were taking a loop diuretic but fewer took ACEI/ARB, β-blocker and statin. (Table 3.1.4) The NT-proBNP, hs-CRP, RDW, D-dimer and vWF activity were higher in those who died compared to the survivors; whilst Albumin, GFR and Hb were lower in those who had died by 5 years.

The uni-variable predictors of all-cause mortality are listed in Table 3.1.5. Of the haemostatic markers, only log(D-dimer) and log(vWF activity) predictors of mortality in uni-variable Cox regression analysis. Both these markers were independent predictor of all-cause mortality when added individually or in combination into the multi-variable Cox model shown in Table 3.1.5. (Table 3.1.6) The improvement in the Chi square of the multi-variable Cox model was minute with the addition of log(vWF activity) and marginally better with the addition of log(D-dimer) alone or in combination with log(vWF activity). (Table 3.1.5 and Table 3.1.6) However, using the same multi-variable model, log(D-dimer) and log(vWF) were independent predictors of cardiovascular death, cardiovascular hospitalisations and combined cardiovascular hospitalisation and all-cause mortality. (Table 3.1.7)

The Kaplan Meier survival curves were plotted using tertiles of D-dimer (Figure 3.1.1) and vWF activity. (Figure 3.1.2)

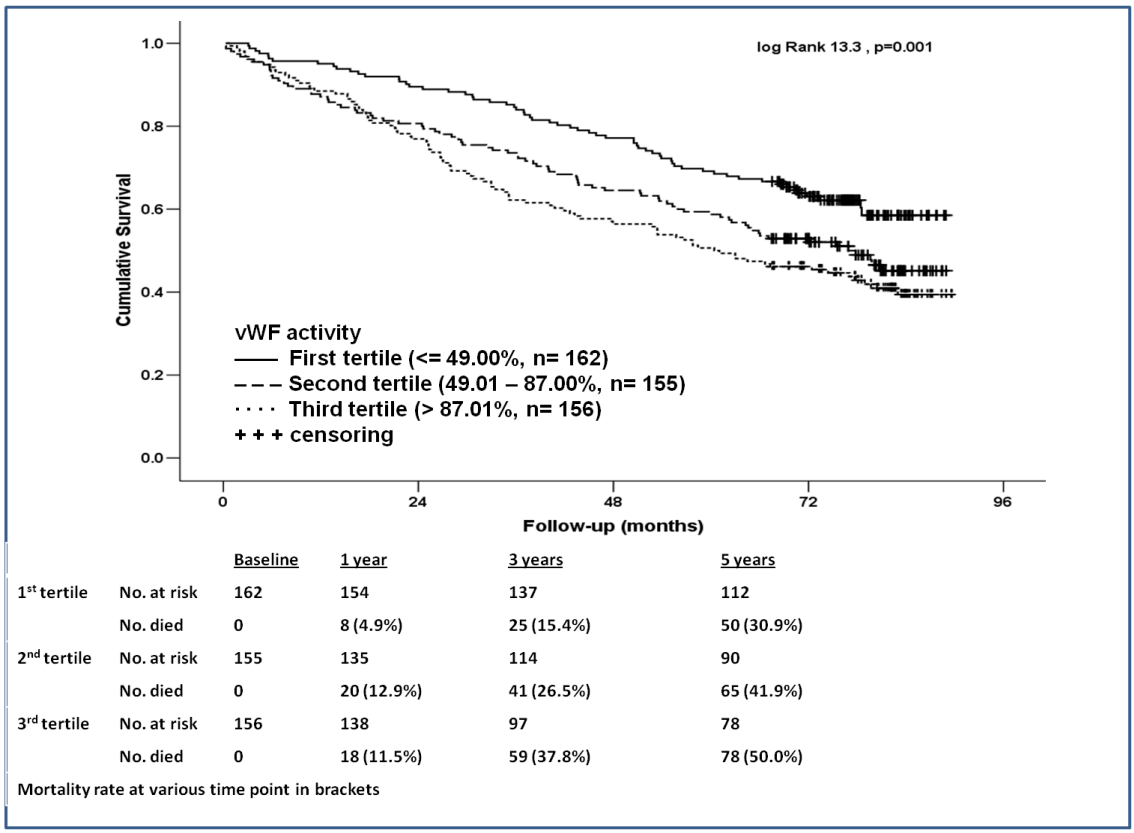
Of the 473 patients, 165 patients returned for repeat blood tests and assessment after a mean of 11.3 ± 1.6 (range 8.0 – 13.7) months following their initial assessment. Of the other 308 patients, 47 had died prior to or during the planned repeat blood sampling period and a further 81 patients did not have adequate blood sample data to be included in the analysis. The remaining 180 patients did not agree for repeat blood tests although they continued to have routine follow-up in the heart failure clinic. Fibrinogen and PAI-1 levels increased but t-PA, sE-Selectin, sP-selectin and vWF activity decreased during repeat testing. The levels of D-dimer, NT-proBNP, Hb, hs-CRP and GFR remained unchanged. (Table 3.1.8)



		Baseline	1 year	3 years	5 years
1st tertile	No. at risk	157	150	134	104
	No. died	0	7 (4.5%)	23 (13.1%)	53 (33.8%)
2nd tertile	No. at risk	159	145	120	101
	No. died	0	14 (8.8%)	39 (24.5%)	58 (36.5%)
3rd tertile	No. at risk	157	132	93	68
	No. died	0	25 (15.9%)	64 (40.8%)	89 (56.7%)

Mortality rate at various time point in brackets

Figure 3.1.1 Kaplan Meier survival curves according to tertiles of D-dimer



		Baseline	1 year	3 years	5 years
1st tertile	No. at risk	162	154	137	112
	No. died	0	8 (4.9%)	25 (15.4%)	50 (30.9%)
2nd tertile	No. at risk	155	135	114	90
	No. died	0	20 (12.9%)	41 (26.5%)	65 (41.9%)
3rd tertile	No. at risk	156	138	97	78
	No. died	0	18 (11.5%)	59 (37.8%)	78 (50.0%)

Mortality rate at various time point in brackets

Figure 3.1.2 Kaplan Meier survival curves according to tertiles of vWF activity

Table 3.1.1 Baseline characteristics of the patients with and without LVSD

	Non-LVSD n=88	LVSD n=473	*p*
Age (years)	68.4 ± 9.5	70.3 ± 10.0	0.06
Men	77.2	77.6	0.895
LVSD			--
Mild-to-moderate	0	24.7	
Moderate	0	43.3	
Severe	0	39.1	
NYHA III/IV	15.2	21.6	0.168
Medical history			
Ischaemic Heart disease	56.5	77.8	<0.001
Diabetes mellitus	14.3	21.9	0.102
Atrial arrhythmias	3.3	30.5	<0.001
Renal dysfunction	3.3	38.1	<0.001
Medication			
ACEI / ARB	38.0	91.3	<0.001
Beta-blockers	43.5	84.4	<0.001
Diuretics	28.3	80.8	<0.001
Aldosterone antagonist	0	27.3	<0.001
Statins	54.3	55.4	0.854
Antithrombotics			<0.001
None	34.1	20.7	
Anti-platelets	62.5	48.4	
Warfarin	2.2	28.1	
Warfarin & anti-platelet	0	2.7	
Laboratory tests			
NT-proBNP (pmol/L)	14.6 (8.0-28.7)	131.5 (53.9-286.6)	<0.001
hs-CRP (mg/L)*	4.5 ± 5.5	7.4 ± 12.6	0.018
D-dimer (ng/ml)	85.7 (40.4-152.0)	104.2 (45.4-237.2)	0.044
Fibrinogen (μg/ml)	63634 (34859-113227)	8233 (4461-15744)	<0.001
t-PA (pg/ml)	1586 (1096-2676)	2526 (1649-3775)	<0.001
PAI-1 (ng/ml)	109.8 (73.3-170.8)	81.3 (54.6-125.1)	<0.001
sP-Sel (ng/ml)	16.8 (13.6-30.4)	31.3 (19.9-46.1)	0.001

sE-Sel (ng/ml)	74.7 (43.4-105.0)	62.5 (44.7-85.2)	0.114
vWF activity (%)	47.0 (31.0-68.8)	67.0 (42.0-103.0)	<0.001
Haemoglobin (g/dL)	14.0 ± 1.4	13.1 ± 1.5	<0.001
WCC ($x10^9$/L)	7.0 ± 1.9	7.3 ± 2.8	0.175
Platelets ($x10^9$/L)	239 ± 67	221 ± 63	0.014
Sodium (mmol/L)	139 ± 3	139 ± 3	0.415
Albumin (g/L)	39 ± 3	38 ± 3	0.003
GFR (mls/min/1.73m^2)	77.5 ± 12.8	54.5 ± 20.5	<0.001

Data are presented as percentage patients or otherwise stated as mean ± standard deviation if normally distributed or median (inter-quartile range) if distribution was not normal

* n=88 in non-LVSD and n=310 in LVSD

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; GFR, glomerular filtration rate; LVSD, left ventricular systolic dysfunction; WCC, white cell count.

Table 3.1.2 The levels of NT-proBNP, GFR, hs-CRP, Hb and haemostatic markers in patients with LVSD according to anti-thrombotic agents

	None n=128	Anti-platelet n=284	Warfarin n=136	Anti-platelet & Warfarin n=13	*p*
NT-proBNP (pmol/L)	126.9 (51.5-456.5)	99.0 (42.8-241.8)	178.4 (97.1-337.3)	199.6 (138.5-421.5)	<0.001
GFR (mls/min/1.73m^2)	51.8 ± 20.5	56.4 ± 20.7	53.5 ± 20.2	52.0 ± 17.3	0.271
hs-CRP (mg/L)*	7.4 ± 9.9	7.4 ± 15.2	7.5 ± 9.9	7.1 ± 6.5	0.238
Haemoglobin (g/dL)	12.9 ± 1.5	13.2 ± 1.4	13.2 ± 1.5	12.6 ± 2.2	0.451
D-dimer (ng/ml)	116.2 (56.9-239.4)	132.9 (69.7-288.2)	51.6 (24.7-142.0)	78.3 (38.2-119.1)	<0.001
Fibrinogen (μg/ml)	6289 (3449-13196)	7515 (3994-15744)	10363 (5753-15845)	17366 (5700-25146)	0.003
t-PA (pg/ml)	2493 (1504-3423)	2628 (1729-3767)	2442 (1602-3719)	3910 (1557-6118)	0.549
PAI-1 (ng/ml)	81.8 (54.2-125.8)	81.3 (56.5-119.8)	81.2 (52.8-125.4)	125.4 (43.8-248.7)	0.688
sP-Sel (ng/ml)	31.3 (25.3-50.3)	31.3 (18.4-41.2)	31.3 (22.9-49.6)	19.2 (10.0-131.1)	0.642
sE-Sel (ng/ml)	66.7 (44.6-92.1)	58.4 (43.3-79.8)	64.5 (48.3-89.2)	67.8 (53.4-107.0)	0.098
vWF activity (%)	70.0 (43.8-106.8)	63.0 (38.0-94.0)	68.0 (44.0-105.5)	69.0 (47.0-143.5)	0.144

Data are presented as mean ± standard deviation if normally distributed or otherwise median (inter-quartile range).

*In total 310 patients had data for hs-CRP and the corresponding number of patients to each of the group above are 71, 146, 84 and 9 patients.

GFR, glomerular filtration rate; PAI-1; plasminogen activator inhibitor-1; sE-Sel, soluble E-Selectin; sP-Sel, soluble P-Selectin; t-PA, tissue plasminogen activator; vWF, von Willebrand factor.

Table 3.1.3 Pearson correlation coefficients among the laboratory variables

	Age	GFR	log(NT-proBNP)	hs-CRP	Haemoglobin	RDW (%)	log(D-dimer)	log(Fibrinogen)	log(t-PA)	log(PAI-1)	log(sP-Selectin)	log(sE-Selectin)	log(vWF activity)
Age (years)	--	-0.44*	0.44*	0.01	-0.20*	0.14*	0.34*	0.04	0.01	-0.22*	-0.03	-0.18*	0.13
GFR (mls/min/1.73m^2)		--	-0.45*	-0.06	0.33*	-0.20*	-0.24*	-0.03	-0.15*	0.12*	0.02	0.02	-0.32
log(NT-proBNP)			--	0.12*	-0.24*	-0.38*	0.24*	0.09	0.06	-0.17*	0.02	-0.09	0.21*
hs-CRP† (mg/L)				--	-0.13*	0.20*	0.08	0.09	0.13*	0.17*	0.01	0.04	0.18*
Haemoglobin (g/dL)					--	-0.29*	-0.10*	-0.01	0.06	0.10*	0.06	0.18*	-0.19*
RDW (%)						--	0.14*	0.06	0.01	0.08	0.02	0.06	0.13*
log(D-dimer)							--	-0.01	0.06	-0.04	-0.01	-0.15*	0.18*
log(Fibrinogen)								--	-0.12*	0.04	0.08	0.01	0.14*
log(t-PA)									--	0.25*	0.02	0.11*	0.09
log(PAI-1)										--	0.08	0.23*	-0.03
log(sP-Selectin)											--	0.16*	-0.01
log(sE-Selectin)												--	-0.01

*2-tailed p<0.001; † n=310 for hs-CRP

Table 3.1.4 Comparison of the characteristics of patients who had died (n=191) and those who were alive (n=282) by 5 years

	Alive n=282	Dead n=191	*p*
Age (years)	67.6 ± 10.0	74.2 ± 8.7	<0.001
Men	79.8	74.9	0.207
LVSD			0.021
Mild-to-moderate	28.4	19.4	
Moderate	44.0	42.4	
Severe	27.7	38.2	
NYHA III/IV	16.3	29.3	0.001
Medical history			
Ischaemic Heart disease	76.2	80.1	0.321
Diabetes mellitus	21.0	23.0	0.598
Atrial arrhythmias	27.3	35.1	0.071
Renal dysfunction	32.3	46.6	0.002
Non-cardiac vascular	17.4	28.8	0.003
Medication			
ACEI / ARB	94.0	87.4	0.013
Beta-blockers	88.3	78.5	0.004
Diuretics	74.8	89.5	<0.001
Aldosterone antagonist	25.9	29.3	0.411
Statins	62.8	44.5	<0.001
Antithrombotics			0.022
None	19.1	23.0	
Anti-platelets	51.8	43.5	
Warfarin	28.0	28.3	
Warfarin & anti-platelet	1.1	5.2	
Laboratory tests			
NT-proBNP (pmol/L)	91.0 (41.0-204.7)	213.5 (106.8-505.6)	<0.001
hs-CRP (mg/L)*	5.9 ± 10.9	9.8 ± 14.4	0.001
D-dimer (ng/ml)	83.0 (38.6-167.7)	158.2 (60.4-299.5)	<0.001
Fibrinogen (μg/ml)	7885 (4293-15539)	8937 (4500-16183)	0.516
t-PA (pg/ml)	2436 (1630-3687)	2713 (1682-3924)	0.225

PAI-1 (ng/ml)	82.7 (54.5-130.1)	79.2 (54.8-121.0)	0.475
sP-Sel (ng/ml)	31.3 (20.9-44.0)	31.3 (17.2-48.6)	0.969
sE-Sel (ng/ml)	62.5 (45.1-85.4)	62.4 (44.1-85.1)	0.751
vWF activity (%)	59.5 (38.0-94.0)	77.0 (49.0-112.0)	<0.001
Haemoglobin (g/dL)	13.4 ± 1.4	12.8 ± 1.5	<0.001
RDW (%)	13.9 ± 1.3	14.6 ± 1.5	<0.001
WCC ($x10^9$/L)	7.0 ± 1.8	7.7 ± 3.8	0.112
Platelets ($x10^9$/L)	222 ± 61	218 ± 65	0.377
Sodium (mmol/L)	140 ± 3	139 ± 3	0.415
Albumin (g/L)	39 ± 3	37 ± 4	0.003
GFR (mls/min/1.73m^2)	59.0 ± 20.7	47.9 ± 18.3	<0.001

Data are presented as percentage patients or otherwise stated as mean ± standard deviation if normally distributed or median (inter-quartile range) if distribution was not normal

* n=187 in alive and n=123 in dead.

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; GFR, glomerular filtration rate; LVSD, left ventricular systolic dysfunction; WCC, white cell count.

Table 3.1.5 Uni-variable and multi-variable Cox regression model for mortality in patients with LVSD

	Uni-variable		Multi-variable	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (years)	1.07 (1.05-1.08)	<0.001	1.04 (1.02-1.06)	<0.001
Man	1.18 (0.87-1.59)	0.292	--	--
NYHA III/IV	2.02 (1.52-2.68)	<0.001	1.26 (0.91-1.76)	0.17
LVSD				
Mild-to-moderate	1.00	0.004	1.00	0.07
Moderate	1.32 (0.93-1.86)		1.07 (0.73-1.56)	
Severe	1.80 (1.26-2.56)		1.49 (0.99-2.24)	
IHD	1.15 (0.84-1.58)	0.369	--	--
Hypertension	1.09 (0.84-1.14)	0.501	--	--
Atrial arrhythmias	1.33 (1.02-1.74)	0.116	0.91 (0.64-1.29)	0.581
Diabetes mellitus	1.12 (0.82-1.51)	0.475	--	--
Non-cardiac vascular disease	1.57 (1.18-2.09)	0.002	1.19 (0.86-1.63)	0.298
ACEI / ARB	0.59 (0.40-0.88)	0.010	0.81 (0.51-1.26)	0.344
ARA	1.18 (0.89-1.56)	0.253	--	--
β-blockers	0.59 (0.43-0.81)	0.001	0.60 (0.43-0.85)	0.004
Loop diuretics	1.91 (1.30-2.80)	0.001	1.40 (0.91-2.15)	0.129
Statins	0.57 (0.44-0.73)	<0.001	0.73 (0.54-0.99)	0.043
Anti-thrombotic		0.001		0.032
None	1		1	
Anti-platelet	0.77 (0.55-1.08)		1.08 (0.74-1.59)	
Warfarin	0.92 (0.64-1.31)		0.91 (0.59-1.42)	
Anti-platelet & warfarin	2.69 (1.43-5.05)		2.47 (1.26-4.86)	
Sodium (mmol/L)	0.96 (0.92-1.00)	0.049	0.99 (0.94-1.04)	0.553
Albumin (g/L)	0.91 (0.88-0.95)	<0.001	1.02 (0.97-1.07)	0.504
GFR (ml/min/1.73m^2)	0.98 (0.97-0.99)	<0.001	1.00 (0.99-1.00)	0.313
Haemoglobin (g/dL)	0.81 (0.75-0.88)	<0.001	0.92 (0.84-1.02)	0.095
White cell count (x 10^9/L)	1.05 (1.01-1.09)	0.007	1.03 (0.99-1.08)	0.200
Platelet (x 10^9/L)	1.00 (1.00-1.00)	0.412	--	--
RDW (%)	1.26 (1.17-1.36)	<0.001	1.05 (0.96-1.16)	0.277
Log_{10}(NT-proBNP)	3.82 (2.93-4.97)	<0.001	2.29 (1.62-3.23)	<0.001

hs-CRP above median*	1.86 (1.34-2.57)	0.001	1.57 (1.09-2.25)	0.014
Log(D-dimer)	1.95 (1.53-2.49)	<0.001		
Log(Fibrinogen)	1.15 90.85-1.54)	0.367		
Log(tPA)	1.31 (0.84-2.04)	0.230		
Log(PAI-1)	0.80 (0.2-1.25)	0.334		
Log(vWF activity)	2.64 (1.69-4.10)	<0.001		
Log(sE-Selectin)	0.86 (0.47-1.57)	0.638		
Log(sP-Selectin)	0.88 (0.65-1.18)	0.388		

Chi square of multi-variable model 190.4, $p<0.001$

*hs-CRP adjusted for missing value

ACEI/ARB, angiotensin converting enzyme inhibitor or angiotensin receptor blocker; ARA, aldosterone receptor antagonist; LVSD, Left ventricular systolic dysfunction; IHD, ischaemic heart disease; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; vWF, von Willebrand factor activity.

Table 3.1.6 Multi-variable Cox regression model for mortality in patients with LVSD with the addition of log(D-dimer) and log(vWF activity) into the model

	log(D-dimer)		log(vWF activity)	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (years)	1.03 (1.01-1.05)	<0.001	1.04 (1.02-1.06)	<0.001
NYHA III/IV	1.32 (0.94-1.83)	0.107	1.22 (0.88-1.71)	0.237
LVSD				
Mild-to-moderate	1.00	0.075	1.00	0.051
Moderate	1.12 (0.77-1.64)		1.07 (0.73-1.58)	
Severe	1.53 (1.02-2.30)		1.54 (1.02-2.24)	
Atrial arrhythmias	0,89 (0.62-1.26)	0.116	0.93 (0.66-1.32)	0.689
Non-cardiac vascular disease	1.16 (0.84-1.60)	0.370	1.24 (0.90-1.71)	0.191
ACEI / ARB	0.82 (0.52-1.28)	0.373	0.87 (0.55-1.37)	0.538
β-blockers	0.60 (0.43-0.85)	0.004	0.60 (0.43-0.85)	0.004
Loop diuretics	1.36 (0.89-2.10)	0.001	1.32 (0.85-2.03)	0.215
Statins	0.69 (0.51-0.95)	0.021	0.72 (0.53-0.98)	0.039
Anti-thrombotic		0.014		0.033
None	1		1	
Anti-platelet	1.04 (0.71-1.52)		1.14 (0.77-1.67)	
Warfarin	1.05 (0.67-1.65)		0.93 (0.59-1.42)	
Anti-platelet & warfarin	2.93 (1.47-5.82)		2.48 (1.25-4.90)	
Sodium (mmol/L)	0.98 (0.94-1.03)	0.491	0.99 (0.94-1.04)	0.583
Albumin (g/L)	1.02 (0.98-1.07)	0.333	1.02 (0.97-1.07)	0.473
GFR (ml/min/1.73m^2)	1.00 (0.99-1.01)	0.434	1.00 (0.99-1.01)	0.741
Haemoglobin (g/dL)	0.90 (0.82-0.10)	0.039	0.92 (0.83-1.01)	0.088
White cell count (x 10^9/L)	1.04 (0.99-1.08)	0.109	1.03 (0.98-1.07)	0.235
RDW (%)	1.05 (0.95-1.16)	0.331	1.06 (0.96-1.17)	0.248
Log_{10}(NT-proBNP)	3.82 (2.93-4.97)	<0.001	2.30 (1.63-3.25)	<0.001
hs-CRP above median*	1.46 (1.02-2.10)	0.039	1.58 (1.10-2.26)	0.012
Log(D-dimer)†	1.48 (1.10-1.99)	0.01	--	--
Log(vWF activity)†	--	--	1.92 (1.17-3.15)	0.01
Chi square of model	196.2	<0.001	192.2	<0.001

*hs-CRP adjusted for missing value.

† When log(DD) and log(vWF activity) were added into the model in combination, the hazard ratios were 1.42 (1.05-1.91), p=0.022 and 1.79 (1.09-2.94), p=0.022 respectively with a Chi square of 197.

ACEI/ARB, angiotensin converting enzyme inhibitor or angiotensin receptor blocker; ARA, aldosterone receptor antagonist; LVSD, Left ventricular systolic dysfunction; IHD, ischaemic heart disease; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; vWF, von Willebrand factor activity.

Table 3.1.7 Hazard ratios for log(D-dimer) and log(vWF activity) in multi-variable Cox regression model for cardiovascular hospitalisation, cardiovascular death and combined cardiovascular hospitalisation and all-cause mortality

	log(D-dimer)		log(vWF activity)	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Cardiovascular hospitalization	1.32 (1.01-1.73)	0.041	1.54 (0.98-2.42)	0.064
Cardiovascular death	1.59 (1.11-2.26)	0.011	2.07 (1.17-3.69)	0.013
Cardiovascular hospitalisation & all-cause death	1.31 (1.04-1.65)	0.025	1.54 (1.03-2.29)	0.035
The variables included in the multivariable Cox regression model used were similar to those in Table 3.1.5 and 3.1.6				

Table 3.1.8 Repeat laboratory tests on NT-proBNP, hs-CRP, Hb and hamostatic markers with estimated GFR (n=165)

	Baseline	Repeat	*p*
NT-proBNP (pmol/L)	128.1 (54.1-251.4)	111.5 (49.3-287.5)	0.477
GFR (mls/min/1.73m^2)	51.9 ± 19.5	52.0 ± 18.6	0.311
hs-CRP (mg/L)*	8.1 ± 16.1	5.9 ± 9.2	0.603
Haemoglobin (g/dL)	13.1 ± 1.4	13.0 ± 1.5	0.111
D-dimer (ng/ml)	89.8 (41.3-206.2)	98.0 (47.2-207.0)	0.817
Fibrinogen (μg/ml)	7099 (4508-12395)	95441 (53753-182360)	<0.001
t-PA (pg/ml)	2526 (1607-3585)	1764 (1180-2832)	<0.001
PAI-1 (ng/ml)	79.6 (52.1-122.9)	99.3 (64.6-134.8)	<0.001
sP-Selectin (ng/ml)	31.3 (12.2-41.8)	21.7 (16.5-32.4)	0.001
sE-Selectin (ng/ml)	58.6 (42.9-80.3)	51.1 (32.5-73.3)	<0.001
vWF activity (%)	71.0 (43.5-106.6)	53.0 (37.6-76.4)	<0.001
*hs-CRP with n=70 patients.			

3.1.4 Discussion

We found that patients with LVSD have disturbed haemostasis, neurohormonal activation, endothelial dysfunction and upregulation of inflammation despite good medical therapy. Of the variables we tested, increasing NT-proBNP, D-dimer and vWF activity were associated with increased mortality.

Our findings are consistent with previous studies showing that HF due to LVSD is associated with a hypercoagulable state.[33,55,197,207,243] The higher D-dimer level in patients with LVSD was accompanied by a lower fibrinogen level suggesting an increase in fibrin generation and thrombogenesis. D-dimer has been consistently shown to be elevated in patients with heart failure.[55,56,207] More recent studies have suggested that elevated D-dimer is a marker of poor prognosis in acute and chronic heart failure. In 214 patients hospitalised with newly diagnosed or decompensated heart failure, after adjustment for factors including NT-proBNP, interleukin-6 and CRP, an elevated D-dimer > 450 ng/ml was independently associated with higher mortality after a mean follow-up period of 8.9 ± 1.9 months.[56] In another study of 458 older patients (65-87 years) with signs and/or symptoms of heart failure, D-dimer > 250 ng/ml was independently associated with all-cause and cardiovascular mortality after adjustment

for BNP and CRP.[57] D-dimer remained an independent predictor after exclusion of patients with atrial fibrillation and renal impairment (Creatinine $\geq$ 200 mmol/L). However, 214 patients in this study had normal systolic and diastolic LV function and the proportions of patient with systolic or 'diastolic' dysfunction were not reported. Our study shows that elevated level of D-dimer (> 121.6 ng/ml) has added prognostic value over conventional predictors, NT-proBNP, hs-CRP and other haemostatic biomarkers in a large group of unselected patients with stable chronic HF due to LVSD.

Increased in thrombogenesis may enhance fibrinolytic activity. We found higher levels of t-PA but lower PAI-1 in patients compared with controls. There are few but conflicting data on fibrinolysis in patients with LVSD and raised D-dimer has been used as an indirect measurement of increased fibrinolysis.[207] However, by measuring plasmin-plasmin inhibitor complex, Yamamoto et al. showed that fibrinolytic activity was similar in patients with idiopathic and hypertrophic cardiomyopathy when compared to normal subjects despite increased thrombogenesis.[209] Another possible explanation for our observation is that angiotensin II and bradykinin stimulate the release of PAI-1 and t-PA respectively. ACEIs may therefore inhibit the release of PAI-1 but increase bradykinin-dependent t-PA release from endothelium.[223] In our study, t-PA was similar in patients who were alive and those who had died by 5 years and it was not a prognostic factor.

Despite having a lower platelet count, patients with LVSD had higher sP-Sel suggestive of increased platelet activation. Similar to previous studies, sP-Sel was not affected by the aetiology and severity of LVSD or the use of anti-platelet or warfarin.[58,197,207,258] Likewise, it was not associated with long-term mortality.

Patients with LVSD also had higher vWF activity but no difference in the level of sE-Sel. vWF and other biomarkers of endothelial dysfunction are elevated in acute and chronic stable heart failure especially in those with worse symptom, LV aneurysm and diabetes.[59,60,202,242] Treatment with ACEI reduces vWF level.[197] However, few data are available with regards to the prognostic importance of these markers in patients with LVSD.[242] In our study, vWF activity but not sE-Sel was higher in patients who had died compared to those who were alive by 5 years and vWF activity was also independent predictors of mortality. However, vWF activity reduced with time whilst D-dimer, NT-proBNP, Hb and hs-CRP levels were static in these patients who were on stable medication suggestive that vWF activity may be a more dynamic and sensitive marker

to detect a change in the clinical state of patients; whilst D-dimer, NT-proBNP and hs-CRP are valid single time point risk markers for stable patients with CHF on optimal therapy.

Patients with LVSD had higher NT-proBNP and hs-CRP consistent with neurohormonal and inflammatory activation. Those with more severe LVSD had higher level of NT-proBNP but not hs-CRP. Both processes are, in part, responsible for disease progression in LVSD. We have shown that hs-CRP has incremental prognostic value over NT-proBNP alone.[69] However, in combination with haemostatic biomarkers, only NT-proBNP, D-dimer and vWF activity were independent predictors of mortality. Nevertheless, it is well established that inflammation can stimulate coagulation via a complex interaction between the two systems.[54] We found hs-CRP only had modest relationship to t-PA, PAI-1 and vWF activity suggestive that factors other than inflammation alone are involved in the activation of coagulation system in CHF. Similar to previous studies, ACEI and/or ARB were associated with lower NT-proBNP and hs-CRP.[372]

Only a small group of patients returned for a repeat blood test. Whilst the levels of NT-proBNP, Hb, hs-CRP and D-dimer were stable, changes in other haemostatic markers were seen. vWF activity and sE-selectin levels decreased with time. As these are markers of endothelial function, such changes may represent an improvement in endothelial function. In addition, t-PA decreased with corresponding increase in PAI-1 indicating a reduction in fibrinolysis. Coupled with an increase in fibrinogen and stable D-dimer level, these observations may be a reflection of decreased thrombogenesis. Further, reduction in sP-selection indicates a lower level of platelet activation supporting the possible reduction in thrombogenic tendency. Indeed, the levels of repeat haemostatic markers in these patients were closer to the 88 controls who did not have LVSD. By the fact that these patients survived and returned for a repeat blood test and follow-up, they may represent a ‘self-selected’ group of patients with lower risk and more stable clinical course. However, the findings are suggestive that, at least, in a group of patients with stable CHF, the thromboembolic risk may reduce with time using established modern CHF therapy even in the absence of intensive anti-thrombotic therapy. This may, partly, explain the relatively low thromboembolic events found in a few randomised controlled studies and the absence of definitive benefit in anti-

thrombotic treatment for patients with CHF who are in sinus rhythm as discussed below.

Perturbed haemostasis may be associated with a higher incidence of thromboembolic events in patients with LVSD as reported in some observational studies and retrospective analysis of randomised controlled trials (RCTs) of treatment of CHF.[38,39,373-377] As many thromboembolic events only become apparent in post-mortem,[47] this may partly explain the relatively low thromboembolic events in the more recent RCTs of anti-thrombotic therapy in HF (WASH, WATCH, HELAS and WARCEF).[46,48,49,51,378] We showed that patients taking warfarin had lower D-dimer and higher fibrinogen levels suggestive of reduction in fibrin generation and thrombogenesis. This may be a possible explanation for the finding that warfarin was associated with fewer HF hospitalisation and a weak trend of lower mortality in the meta-analysis of WASH and WATCH.[48,379] However, the reason that aspirin was associated with increase HF hospitalisation is unclear as it did not alter the level of biomarkers investigated in our study. In WARCEF, only 29/1142 patients taking warfarin and 55/1163 patients taking aspirin had an ischaemic stroke after 3.5 ± 1.8 years with warfarin being associated with a lower rate of stroke when compared to asprin.[53] Patients younger than 60 years old may have a net benefit from warfarin therapy when compared to those taking aspirin with acceptable risk of bleeding but lower combined death and ischaemic and haemorrhagic stroke.[52] It is possible that D-dimer may be a marker to identify the appropriate patients for anti-coagulation therapy.

An interesting finding is that statin prescription was an independent predictor of better outcome in this study. This is not consistent with two large randomised controlled trials that have shown neutral effect of statins on the prognosis of patients with CHF.[380,381] However, subgroup of patients with CHF and lower NT-proBNP[382] or higher hs-CRP[383] may benefit from rosuvastatin. In this study, patients taking a statin had lower NT-proBNP [102.0 (43.0-206.2) vs. 169.6 (84.0-386.1) pmol/L, $p< 0.001$] and trended to have lower hs-CRP (7.3 ± 14.6 vs. 7.5 ± 9.8 mg/L, $p=0.051$) than those who were not. We have also previously shown that in clinical setting, patients with LVSD and taking a statin have better survival than those who are not taking a statin or have their statin stopped for various reasons.[384]

3.1.4.1 Limitations

There was no age- and sex-matched normal controls in our study. However, given that various disease processes including renal impairment, non-cardiac vascular disease, atrial arrhythmias and diabetes can affect the level of these biomarkers, comparing LVSD patients to non-LVSD patients with established or at risk of developing cardiovascular disease would give clearer picture on the effects of LVSD on these markers. Patients with atrial fibrillation and renal impairment were included since these conditions are common in patients with LVSD. Hypercoagulation exists in LVSD patients even in the absence of atrial fibrillation.[197] Importantly, there was no difference in all of the haemostatic markers measured except a lower D-dimer in patients with atrial fibrillation and this can be explained by warfarin therapy.[379] A previous study has also shown that exclusion of patients with atrial fibrillation and renal impairment does not affect the prognostic value of D-dimer.[57] Further all regression analyses in this study were adjusted for GFR and atrial dysrrhythmias. We were unable to perform hs-CRP in some patients due to unavailability of the assay for a period of time within the local hospital laboratory but all relevant analyses had been adjusted for this factor. In addition, not every surviving patient returned for a repeat blood sample within the proposed time period leaving a much smaller cohort of patients with repeat blood sampling.

3.1.5 Conclusion

Patients with chronic heart failure due to LVSD have increased thrombogenesis and platelet activation, endothelial dysfunction, neurohormonal activation, and upregulation of inflammation despite optimal medical therapy. Higher D-dimer level and vWF activity are associated with greater mortality and this cannot be explained by other known prognostic factors including NT-proBNP and hs-CRP. D-dimer may help risk stratification in patients with heart failure and patient selection in clinical studies, particularly those involving anti-thrombotic therapy.

3.2 The effect of heart failure treatment optimisation on haemostatic markers in patients with heart failure due to left ventricular systolic dysfunction.

3.2.1 Introduction

Patients with CHF due to LVSD have perturbed haemostasis.[32] However, conventional anti-thrombotic therapy such as aspirin, clopidogrel or warfarin have not been convincingly shown to alter the course of the disease and the prognosis of patients with CHF.[33,34,46,48,51,192] We have also shown that some of the haemostatic markers may confer short- and long-term prognostic information in patient with CHF on stable heart failure treatment.[370,371] The level of these markers change over time even despite stable medications and consistent with a lesser extent of haemostatic disturbance with improvement in endothelial function.

The Warfarin Versus Aspirin in Patients With Reduced Cardiac Ejection Fraction (WARCEF) randomised 2305 (instead of initial target of 2860) patients with LVEF <= 35% without atrial fibrillation or mechanical prosthetic valve to receive warfarin compared aspirin with a composite primary end-point of death and ischaemic or haemorrahgic stroke.[385] Although the outcome was neutral, post-hoc analysis suggests that patients younger than 60 years may have a net benefit of lower long-term composite end point without offsetting by the associated risk of bleeding.[51,52] Further, the main benefit of warfarin therapy was associated with the reduction in ischaemic cardioembolic stroke.[53] Although clinically apparent thromboembolic events are relatively low, post-mortem study has shown a high prevalence of thromboembolic events in patients with LVSD.[47] Therefore, haemostatic markers may identify patients at higher risk of thromboembolic events so that such patients can be targeted for treatment.

However, it is unclear if standard modern heart failure treatment may alter the haemostatic disturbance in patient with CHF is unclear. Therefore, in a group of ambulatory patients with newly diagnosed HF due to left ventricular systolic dysfunction (LVSD) undergoing initiation or optimisation of their treatment, we investigated the effect of treatment on the levels of haemostatic biomarkers and NT-proBNP.

3.2.2 Methods

3.2.2.1 Patients

Patients who were found to have CHF due to LVSD following assessment in the heart failure service in Hull and East Yorkshire Hospitals NHS Trust and those referred for initiation or optimisation of their treatment following the diagnosis of HF due to LVSD were recruited. Those who had an episode of decompensated heart failure or acute coronary syndrome within the previous 4 weeks were excluded. All patients gave informed written consent.

3.2.2.2 Investigations

These patients were assessed and underwent investigations including blood tests, electrocardiogram and echocardiogram at baseline and during follow-up as previously described in Chapter 3.1.2.2. Specifically, blood samples were collected for analysis of NT-proBNP, D-dimer, fibrinogen, von Willebrand factor (vWF) activity, soluble E-selectin (sE-selectin), soluble P-selectin (sP-selectin), tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) in the core laboratory as described earlier. The changes in these markers were studied.

3.2.2.3 Follow-up

The treatment strategy was to achieve optimal heart failure therapy for all the patients within the first four months of their initial presentation to the clinic. The patients were reassessed three months after achieving stable and optimal heart failure therapy as described above. These patients were then followed up four monthly for the first year from their initial presentation and then annually, unless more frequent visit was deemed necessary.

3.2.2.4 Statistical analysis

Data presentations and statistical analyses were done as described in Chapter 2.3 and 3.1.2.4

3.2.3 Results

A total of 66 patients of whom 46 (69.7%) were men with a mean age of 71.4 ± 8.0 years were included in this analysis. (Table 3.2.1)

Table 3.2.1 Baseline characteristics (n=66)

	n=66
Age (years)	71.4 ± 8.0
Men	69.7
NYHA III/IV	28.8
LVSD	
Mild	27.3
Moderate	40.9
Severe	31.8
Ischaemic heart disease	81.8
Diabetes mellitus	18.2
Hypertension	53.0
Atrial arrhythmias	25.8
Non-cardiac vascular disease	25.8
LVSD, left ventricular systolic dysfunction; NYHA, New York Heart Association breathlessness classification.	

These patients returned for a repeat assessment and blood tests after a mean of 5.1 ± 2.2 (range 3.2 – 14) months. At follow-up, higher proportion of patients were taking ACEI, ARB and β-blocker and at a more optimal dosage but diuretic prescription did not change. (Table 3.2.2) More patients were also taking warfarin instead of anti-platelets.

Table 3.2.2 Changes in medication, symptom, LV function and labaoratory tests following heart failure treatment optimisation

	Baseline (n = 66)	Reassessment (n = 66)	*P*
NYHA III/IV	28.8	22.7	0.523
LVSD			0.506
Mild-to-moderate	27.3	27.3	
Moderate	40.9	43.9	
Severe	31.8	28.8	
Loop diuretic	80.3	80.3	1.00
Furosemide equivalent dose (mg)	57 ± 25	65 ± 44	0.136
ACEI	50.0	72.7	0.003
Percentage maximum ACEI dose (%)	29 ± 38	46 ± 41	0.002
ARB	4.5	15.2	0.016
Percentage maximum ARB dose (%)	2 ± 12	11 ± 30	0.007
ACEI/ARB	54.5	87.9	<0.001
ARA	18.2	24.2	0.388
β-blocker	59.1	81.8	<0.001
Percentage maximum β-blocker dose (%)	27 ± 36	44 ± 37	<0.001
Statin	51.5	53	1.00
Anti-platelet	62.1	54.5	0.267
Warfarin	19.7	31.8	0.008
Sodium (mmol/L)	140 ± 3	140 ± 3	0.246
GFR (ml/min/1.73m^2)	55.4 ± 20.5	52.8 ± 22.0	0.029
Albumin (g/L)	38 ± 3	38 ± 4	0.906
Haemoglobin (g/dL)	13.4 ± 1.7	13.0 ± 1.6	0.063
White cell count (x10^9/L)	7.2 1.8	6.9 1.7	0.360
Platelets (x10^9/L)	236 61	226 60	0.225
RDW (%)	14.9 ± 1.7	14.3 ± 1.2	0.009

NT-proBNP (pmol/L)	237.1 (92.6-524.3)	115.7 (61.0-375.3)	0.006
hs-CRP (mg/L)	4.6(1.9-11.3)	6.2 (3.1-14.0)	0.385
D-dimer (ng/ml)	143.3 (69.6-355.6)	119.9 (57.6-289.3)	0.041
Fibrinogen (ug/ml)	60222 (23842-115391)	11293 (4131-16380)	<0.001
t-PA (pg/ml)	2305 (1517-2840)	2704 (1855-3666)	0.101
PAI-1 (ng/ml)	98.5 (67.5-125.8)	84.8 (63.5-127.6)	0.181
sP-selectin (ng/ml)	27.2 (9.7-55.0)	31.3 (15.9-47.1)	0.394
sE-selectin (ng/ml)	69.3 (44.7-82.4)	64.3 (50.2-55.0)	0.308
vWF activity (%)	47.2 (30.7-84.8)	63.0 (42.0-89.8)	0.055

Continuous data are presented in mean ± standard deviation if normally distributed and median (inter-quartile range) if otherwise. Categorical data are in percentage of patients. ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; GFR, glomerular filtration rate; hs-CRP, high-sensitivity c-reactive protein; LVSD, left ventricular systolic dysfunction; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association breathlessness classification; PAI-1, plasminogen activator inhibitor-1; RDW, red cell distribution width, t-PA, tissue plasminogen activator; vWF, von Willebrand factor.

The NYHA class and severity of LVSD did not change during the follow-up period although the NT-proBNP level had decreased. (Table 3.2.2) D-dimer and fibrinogen levels reduced but the other haemostatic markers did not change during repeat testing. There was a minor but significant decreased in the GFR during follow-up.

Whether the patients were taking a β-blocker or angiotensin converting enzyme inhibitor / angiotensin receptor blocker (ACEI/ARB) did not affect the laboratory markers at baseline. (Table 3.2.3) Following optimisation of heart failure treatment, Fibrinogen increased in all subgroups of patients regardless of initial treatment regimen. The only other changes in the biomarkers were reduction in NT-proBNP and increased vWF activity in the patients who were not taking a b-blocker or ACEI/ARB. Antithrombotic therapy (Table 3.2.4) and severity of LVSD at baseline did not affect the levels of these haemostatic markers at baseline or follow-up.

Table 3.2.3 Changes between baseline and follow-up blood results according to ACEI/ARB and β-blocker

		No BB No ACEI/ARB n= 12	No BB ACEI/ARB n=15	BB No ACEI/ARB n=18	BB ACEI/ARB n=21
Anti-thrombotics *BL					
None		16.7	33.3	11.1	19.0
Anti-platelets		83.3	40.0	77.8	47.6
Warfarin		0	26.7	11.1	28.6
Combined		0	0	0	4.8
Anti-thrombotics *FU					
None		16.7	26.7	16.7	9.5
Anti-platelets		66.7	40.0	29.4	47.6
Warfarin		16.7	33.3	27.8	33.3
Combined		0	0	0	9.5
Furosemide	BL	91.7	86.7	77.8	71.4
	FU	91.7	86.7	83.3	66.7
Furosemide	BL	47.3 ± 16.2‡	60.0 ± 28.3	50.8 ± 21.0	66.0 ± 30.2
Dose eq. (%)	FU	66.7 ± 35.5‡	41.3 ± 24.5	72.2 ± 62.5	44.8 ± 49.8
ACEI	BL	0	93.3	0	90.5
	FU	83.3	86.7	50.0	76.2
ACEI	*BL	0†	63.3 ± 41.9	0	45.4 ± 33.7
dose eq. (%)	FU	54.2 ± 40.0†	65.0 ± 41.0	22.6 ± 33.2	47.9 ± 41.4
ARB	BL	0	6.7	0	9.0
	FU	8.3	13.3	27.8	9.5
ARB	BL	0	6.7 ± 25.8	0‡	0.6 ± 2.8
dose eq. (%)	FU	8.3 ± 28.9	13.3 ± 35.2	20.8 ± 38.6‡	21.0 ± 2.4
ACEI/ARB	BL	0	100	0†	100
	FU	91.7	100	77.8†	85.7
β-blocker	BL	0	0	100	100
	FU	50.0	60.0	100	100
β-blocker	*BL	0‡	0‡	47.2 ± 41.2‡	42.9 ± 31.3‡
dose eq. (%)	*FU	21.9 ± 31.1‡	22.5 ± 30.3‡	64.6 ± 36.2‡	53.6 ± 30.7‡
ARA	BL	16.7	13.3	11.1	28.6
	FU	33.3	26.7	16.7	23.8
NT-proBNP	BL	365 (84-524)‡	147 (60-385)	700 (120-1216)	218 (93-449)
(pmol/L)	FU	67 (54-99) ‡	110 (67-407)	238 (77-745)	120 (50-255)

hs-CRP	BL	8.7 (2.5-19.3)	3.8 (1.5-6.8)	5.4 (3.0-21.3)	2.7 (1.6-7.7)
(mg/L)	FU	6.1 (4.0-13.0)	2.6 (0.3-11.6)	82. (5.5-19.8)	5.5 (2.8-10.5)
D-Dimer	BL	166 (116-323)	105 (84-312)	116 (51-632)	102 (46-340)
(ng/ml)	FU	123 (88-188)	115 (46-191)	139 (52-381)	122 (52-363)
Fibrinogen	BL	39829‡	82988†	62794†	62748†
(μg/ml)		(19890-103816)	(946935-145501)	(22799-89354)	(23119-141730)
	FU	7634‡	11733†	13807†	11240†
		(4308-22794)	(5125-15045)	(3894-26690)	(3822-16047)
t-PA	BL	2350	2396	2278	2068
(pg/ml)		(1087-2975)	(1537-2800)	(1609-2666)	(1649-3300)
	FU	2282	2747	2622	3101
		(1220-3030)	(1919-4079)	(1607-2899)	(2468-4901)
PAI-1	BL	101 (68-126)	99 (63-129)	101 (82-117)	98 (66-132)
(ng/ml)	FU	72 (59-160)	103 (65-119)	85 (64-102)	86 (64-137)
sP-selectin	BL	33 (13-57)	45 (14-94)	28 (16-52)	18 (1-39)
(ng/ml)	FU	31 (8-31)	31 (7-50)	31 (17-72)	31 (22-62)
sE-selectin	BL	72 (52-84)	61 (29-80)	70 (45-76)	60 (36-90)
(ng/ml)	FU	75 (52-100)	64 (48-84)	63 (52-93)	64 (50-81)
vWF activity	BL	44 (25-79) ‡	47 (31-84)	62 (34-100)	44 (26-69)
(%)	FU	66 (38-121) ‡	59 (37-147)	67 (50-87)	57 (41-74)

* $p<0.05$ comparing antithrombotic subgroups; † $p<0.01$ comparing baseline and follow-up within each subgroup; ‡ $p < 0.05$ comparing baseline and follow-up within each subgroup.

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker, BB, β-blocker; BL, baseline; FU, follow-up; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue plasminogen activator; vWF, von Willebrand factor

Table 3.2.4 Changes between baseline and follow-up blood results according to anti-thrombotic therapy

Baseline anti-thrombotics		None n= 11	Anti-platelets n=34	Warfarin n=19	Combined n=2
Anti-thrombotics *FU					
None		38.5	15.0	0	0
Anti-platelets		23.1	77.5	0	0
Warfarin		38.5	7.5	91.7	0
Combined		0	0	8.3	100
Furosemide	BL	84.6	77.5	83.3	100
	FU	76.9	82.5	75.0	100
Furosemide	BL	45 ± 12	57 ± 24‡	34 ± 20	40
Dose eq. (%)	FU	35 ± 26	65 ± 54‡	48 ± 43	40
ACEI	*BL	61.5	35.0	83.3	100
	FU	84.6	65.0	83.3	100
ACEI	BL	20 ± 28†	25 ± 39	50 ± 41	25
dose eq. (%)	FU	61 ± 43†	39 ± 41	53 ± 40	50
ARB	BL	7.7	5.0‡	0	0
	FU	7.7	20.0‡	8.3	0
ARB	BL	1 ± 3	3 ± 16‡	0	2 ± 12
dose eq. (%)	FU	2 ± 7	15 ± 34‡	8 ± 29	11 ± 29
ACEI/ARB	*BL	69.2	40.0†	83.3	100
	FU	92.3	85.0†	91.7	100
β-blocker	BL	46.2	60.0†	66.7	100
	FU	69.2	82.5†	91.7	100
β-blocker	BL	31 ± 42	27 ± 36†	20 ± 27	50
dose eq. (%)	FU	42 ± 43	45 ± 36†	42 ± 34	50
ARA	BL	7.7	17.5	33.3	0
	*FU	15.4	17.5	58.3	0
NT-proBNP	BL	211 (89-395)	256 (94-589)†	342 (54-606)	882
(pmol/L)	FU	232 (71-422)	78 (50-253)†	221 (134-611)	120
hs-CRP	BL	8.7 8.4	9.3 11.0	3.8 2.6	20.0
(mg/L)	FU	8.2 6.1	15.8 27.7	5.8 5.2	5.5
D-Dimer	BL	227 (93-334)	119 (51-357)	170 (76-460)	525

(ng/ml)	FU	113 (47-142)	158 (56-447)	121 (62-182)	81
Fibrinogen (μg/ml)	BL	82988† (20811-132801)	52516† (22950-105299)	60222† (33278-108818)	192456
	FU	4860 (3217-11106)	10545† (4040-16203)	13196† (11419-222-3)	15700
t-PA (pg/ml)	BL	1848† (1205-2823)	2299 (1394-2634)	2919 (1986-5673)	3832
	FU	2438 (1688-4225)	2704 91893-3555)	2885 (1934-6870)	3910
PAI-1 (ng/ml)	BL	103 (66-130)	97 (74-123)	113 (58-144)	132
	FU	98 (63-148)	74 (61-111)	96 (64-127)	127
sP-selectin (ng/ml)	BL	27 (13-76)	28 (2-59)	18 (9-38)	28
	FU	31 (31-39)	31 912-52)	29 (7-49)	390
sE-selectin (ng/ml)	BL	74 (33-81)	68 (47-81)†	65 (47-93)‡	26
	FU	64 (41-85)	74 (57-99)†	59 (27-73)‡	82
vWF activity (%)	BL	47 (33-87)	55 (30-94)	37 (30-67)‡	57
	FU	42 (9-72)	67 (49-94)	66 (49-136)‡	69

* $p<0.05$ comparing antithrombotic subgroups; † $p<0.01$ comparing baseline and follow-up within each subgroup; ‡ $p < 0.05$ comparing baseline and follow-up within each subgroup. ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker, BB, β-blocker; BL, baseline; FU, follow-up; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue plasminogen activator; vWF, von Willebrand factor

3.2.4 Discussion

Following initiation and optimisation of heart failure treatment in ambulatory patients with newly diagnosed or identified CHF due to LVSD, this analysis showed a reduction in NT-proBNP, D-dimer and fibrinogen levels with a minor reduction in the GFR.

The reduction in D-dimer observed in this study is likely to be related to the higher rate of warfarin prescription following optimisation of treatment regimen and is consistent with the prevalence of atrial arrhythmias within the study cohort. In patients with CHF, warfarin reduces D-dimer, thrombin/antithrombin III complexes and prothrombin fragment F1 + 2.[202,210] This effect is seen within a month following

introduction of warfarin therapy.[210] The reduction in D-dimer is a reflection of decrease thrombogenesis and may explain the potential benefit of warfarin therapy in reducing cardioembolic stroke in younger patients with CHF who are in sinus rhythm as reported in the recent WARCEF study.[52,53] The reduction D-dimer may suggest a better prognosis as we have shown earlier.[370,371]

Intuitively, with the reduction in thrombogenesis as reflected by a lower D-dimer level, one would expect a higher fibrinogen level as fibrinogen is known to be raised in patients with CHF.[60,386] However, Lip et al. has shown that warfarin did not affect fibrinogen level in patients with LVSD despite a reduction in D-dimer.[202] In the contrary, we observed a reduction in fibrinogen level in the patients following optimisation of heart failure treatment. In 20 patients with CHF and LVEF <= 40% who were in sinus rhythm, Gibbs et al. has shown that fibrinogen level but not sP-selectin decreased following introduction of lisinopril.[197] In the same study but a separate cohort of 20 patients, treatment with either carvedilol or bisoprolol did not alter the level of fibrinogen. Hence, the reduction in fibrinogen observed in this study may be due to the effect from ACEI. How ACEI can affect the level of fibrinogen is unknown but the reduction it may be one of the mechanism(s) that contribute to the beneficial effects of ACEI especially in those with CHF.

The reduction in fibrinogen in the patients may partly lead to a reduction in D-dimer seen in this study. Fibrinogen is a main factor that promotes fibrin formation and a determinant of plasma viscosity. Raised level of fibrinogen can lead to abnormal rheology and a pro-thrombotic state with associated cardiovascular complications and increased risk of long-term cardiovascular death.[204] Indeed, we have shown that fibrinogen level is increased in patients with advanced CHF and cardiac cachexia.[201] As fibrinogen is also an acute phase protein, the raised level may be partly related to increased liver synthesis in response to inflammatory activation in CHF.[201]

Both t-PA and PAI-1 levels are raised in patients with heart failure due to LVSD or PSEF but its prognostic value is unknown.[205,206] t-PA antigen level is also an independent predictor of heart failure-related death and hospitalization patient with stable CHF patients.[205] Both ACEI and ARB can increase the level or activity of t-PA but reduce the level or activity of PAI-1 in patients with CHF.[224,236,387] Their effects may be mediated through a quicker and more direct antagonism by ARB on angiotensin II type 1 (AT_1) receptor or slower bradykinin-related t-PA release by ACEI.[223,224,236] Certain β-blockers such as carvedilol can also increase the level of t-PA and decrease

PAI-1.[228] On the other hand, patients with CHF taking warfarin have higher level of active PAI-1 than those who are not but t-PA level is not affected by warfarin.[198] Although statistically insignificant, we observed a reduction in the PAI-1 level with an increased t-PA following initiation or optimisation of heart failure treatment in the patients within this study.

Similar to a previous study using lisinopril, carvedilol or bisoprolol, we did not observe any change in the level of sP-selectin level.[197] The same study showed that lisinopril but not β-blockers reduced vWF activity after 3 to 6 months of treatment. However, another larger and longer-term follow-up study suggests that carvedilol but not metoprolol may reduce vWF activity after 1 year with sustained reduction at 2 years.[228] In contrast, the vWF activity of the patients in this study showed a trend to increase with heart failure treatment. This is unlikely to be related to warfarin therapy as warfarin has been shown to have neutral effect on vWF.[202] The reason for the change in vWF level is unclear but may be related to the small number of patients since sE-selectin, another marker of endothelial function, did not change.

3.2.4.1 Limitations

The study is small and initiation or optimisation of heart failure medications was done on different agents simultaneously and so the effect on individual agent has on the haemostatic makers could not be clarified. However, only 12 patients were truly 'naive' of either ACEI/ARB or β-blocker treatment in this study. This reflects the improvement in evidence-based practice by clinicians such that prognostically important medications are being introduced early, for example, following acute coronary syndrome and before the onset or identification of LVSD. In addition, the introduction of warfarin therapy was based on clinical need and not delayed for the purpose of this study as it was unethical to do so.

3.2.5 Conclusion

Initiation and optimisation of standard heart failure therapy including b-blocker and ACEI/ARB and appropriate introduction of warfarin therapy in patients with CHF due to LVSD can reduce D-dimer and fibrinogen levels with the associated reduction in NT-proBNP suggestive of an improvement in the neurohormonal and haemostatic profile of the patients.

Chapter 4 Red and white cell variables in chronic heart failure

Relation between Variables Obtained from a Routine Full Blood Count and Prognosis in Patients with Chronic Heart Failure due to Left Ventricular Systolic Dysfunction

4.1 Introduction

The prevalence of CHF continues to grow due to the improved survival of patients with CHF, and partly due to the aging population.[388,389] Simple, inexpensive methods that help stratify risk may help guide patient management and the planning of health service. Also, there may be a biological reason for the statistical association observed between a variable and outcome which could be a therapeutic target.

B-type natriuretic peptide (BNP) and NT-proBNP are widely used diagnostic and prognostic biomarkers in patients with CHF.[166-168] Natriuretic peptides might also be used as a therapeutic target by which to guide treatment but this remains controversial.[166-168] Other widely investigated biomarkers such as hs-CRP and troponins may have added prognostic value over BNP but their contribution is modest and at an extra cost.[67,69,176,390]

The FBC, which includes measurement of red and white cell variables, is an inexpensive test that should be done routinely all patients with CHF. Both anaemia and red cell distribution width (RDW) provide prognostic information in addition to NT-proBNP.[7,73]

White cell variables are also associated with prognosis, perhaps because they may reflect inflammation. In patients with acute or advanced (but stable) CHF, a raised white cell count (WCC) or neutrophil count is associated with a worse prognosis,[7,141,148] as is a lower lymphocyte count or relative lymphocyte count (where relative lymphocyte count (RLC) is the ratio of lymphocytes to total WCC).[142-144,147] The ratio of neutrophils to lymphocytes (NLR) may be a better predictor of outcome than other white cell variables in patients with acute heart failure,[141] with higher neutrophil count relative to lymphocytes being associated with a worse prognosis, but its value in patients with *chronic* heart failure is unknown.

We investigated the relation between variables available from a FBC and prognosis in patients with CHF.

4.2 Methods

Consecutive patients referred to a community CHF clinic with suspected heart failure between 1st January 2000 to 4th July 2011 were included if they had LVSD equivalent to left ventricular ejection fraction (LVEF) < 45% on echocardiography and if a FBC and plasma NT-proBNP were available. LVEF and degree of LVSD was assessed and classified as described in Chapter 2.2.2. Patients with malignancy, haematological disorders, active infection, inflammatory or auto-immune diseases and taking immunosuppressants including corticosteroid were excluded from the analysis.

All patients were assessed and underwent investigations as described in Chapter 2.2.2 and 2.2.3. The FBC was performed using a commercially automated system (XE 2100 auto-analyser, Sysmex Corporation, Kobe, Japan). NLR was the neutrophil-to-lymphocyte ratio, RLC the ratio of lymphocytes to total WCC and RNC the ratio of neutrophils to total WCC.

Patients were enrolled at their initial clinical assessment after which treatment was optimized. Patients had routine clinic visits at four months, one year and then annually. All-cause mortality was the outcome of interest and vital status was known for all the patients at the censor date on 2nd December 2011.

4.3 Statistical analysis

Variables were presented, analysed, tested for normal distribution and compared as described earlier in Chapter 2.3.

Receiver Operating Characteristic (ROC) curves were used to determine the area under the curve (AUC) for each FBC variable in predicting one-year mortality. Correlation between variables was performed using Pearson's correlation coefficient and scatter plots. Collinearity among variables was examined by Tolerance and Variance Inflation Factor (VIF). Uni-variable Cox regression analysis was used to identify variables associated with mortality as described in Chapter 2.3. To identify independent predictors of long-term mortality, forward stepwise multi-variable Cox regression analysis was performed using all variables identified from the uni-variable analysis except the white cell variables due to the presence of multi-collinearity (defined as VIF > 2.5) or strong correlation. Cox models were constructed with each of the white cell variables entered separately. The c-statistics of the multi-variable Cox model before and after the addition of the blood count variables were calculated in order to see if any added prognostic value. To illustrate the relation between variables and outcome, we

constructed Kaplan-Meier plots using the best cut-offs derived from ROC curve analysis to group the patients. The curves were adjusted for log[NT-proBNP] and other independent predictors identified from multi-variable Cox analysis.

4.4 Results

Of the 2019 patients, 119 were excluded from this analysis due to concomitant cancer (n=34), haematological disorders (n=16), inflammatory diseases or infection (n=64) or a combination of these diseases (n=5). The remaining 1900 patients had a median follow-up period of 64.8 (5.0 – 96.7) months, during which 878 (46.2%) patients died. The baseline characteristics of all the patients are shown in Table 4.4.1.

The blood count variables did not have strong relationships to other clinical or laboratory variables including NT-proBNP. There were only modest correlations between hs-CRP (n = 327) and the white cell variables. Red cell variables were only moderately related to the white cell variables; whilst the white cell variables were more strongly related to each other. (Table 4.4.2 and Table 4.4.3)

Those patients who died during the firsr year (n=197) had higher NT-proBNP, RDW, WCC, NLR, neutrophil count and RNC but lower Hb, lymphocyte count and RLC compared to the survivors (n = 1609). (Table 4.4.1) The ROC curves for 1-year mortality are shown in Figure 4.4.1 (Table 4.4.4)

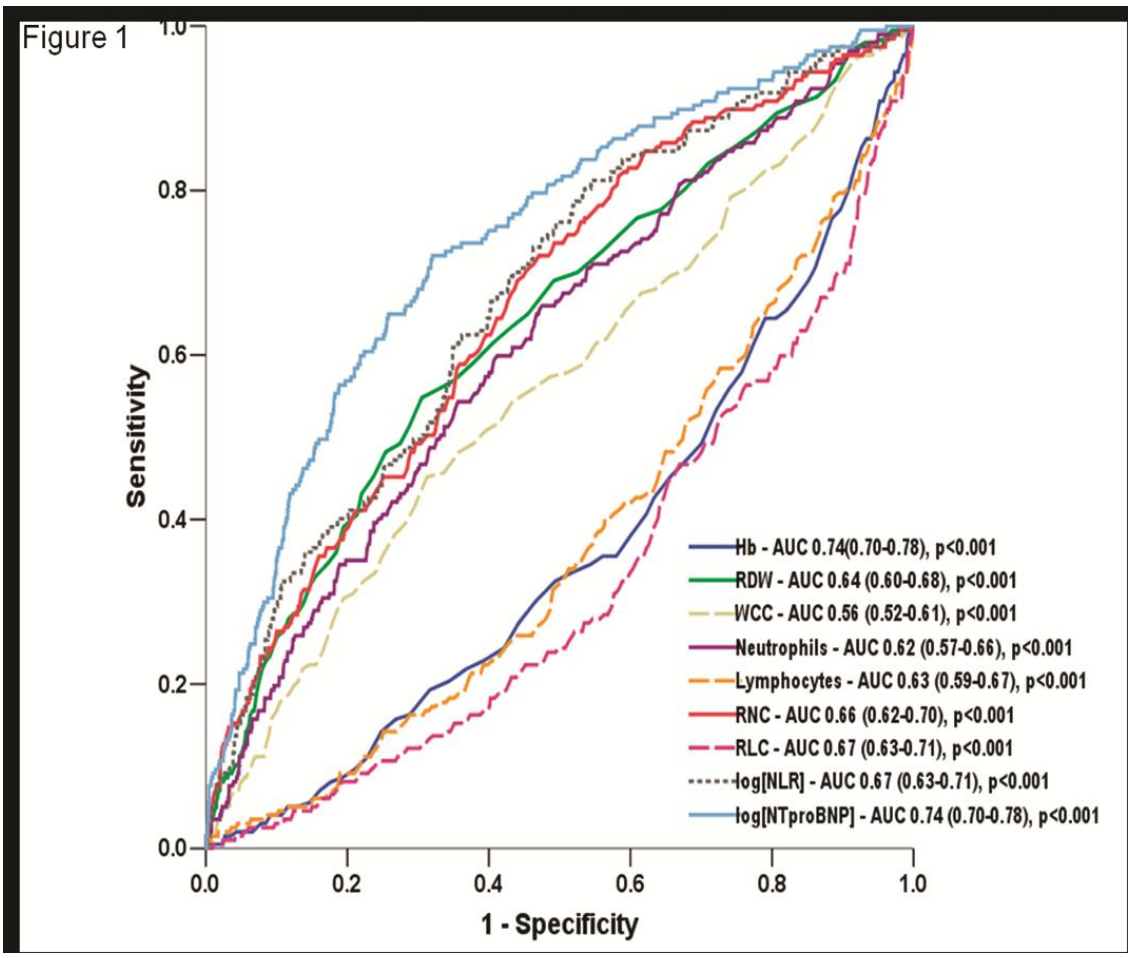


Figure 4.4.1 Receiver operator characteristic curves of NT-proBNP and red and white cell variables in predicting 1-year mortality in the patients who had completed at least one year follow-up (n=1806). Haemoglobin (Hb) and relative lymphocyte count (RLC) has an inverse relationship to mortality.

Many of the variables from the FBC predicted mortality in uni-variable Cox regression analysis. (Table 4.4.5) Independent predictors were older age, worse NYHA functional class, the presence of IHD and other vascular diseases, higher log[NT-proBNP], urea and log[NLR], and lower diastolic blood pressure and sodium. There were strong correlations between many of the white cell variables, so they were included separately in multi-variable Cox model. (Table 4.4.5) RDW and white cell variables (other than lymphocyte count) were independent predictors of mortality.

The c-statistic of the multivariable Cox model increased with the addition of log[NT-proBNP] to a base model. There were further small increments in the c-statistic with the separate addition of RDW, WCC, neutrophil count, RNC, RLC or log[NLR] to the model individually; the greatest increment was seen when RDW was added in combination with a white cell variable. (Table 4.4.6)

The adjusted survival curves showed that combination of RDW and a white cell variable provided incremental prognostic information addition to other variables identified from the Cox model including log[NT-proBNP]. (Figure 4.4.2 a – e)

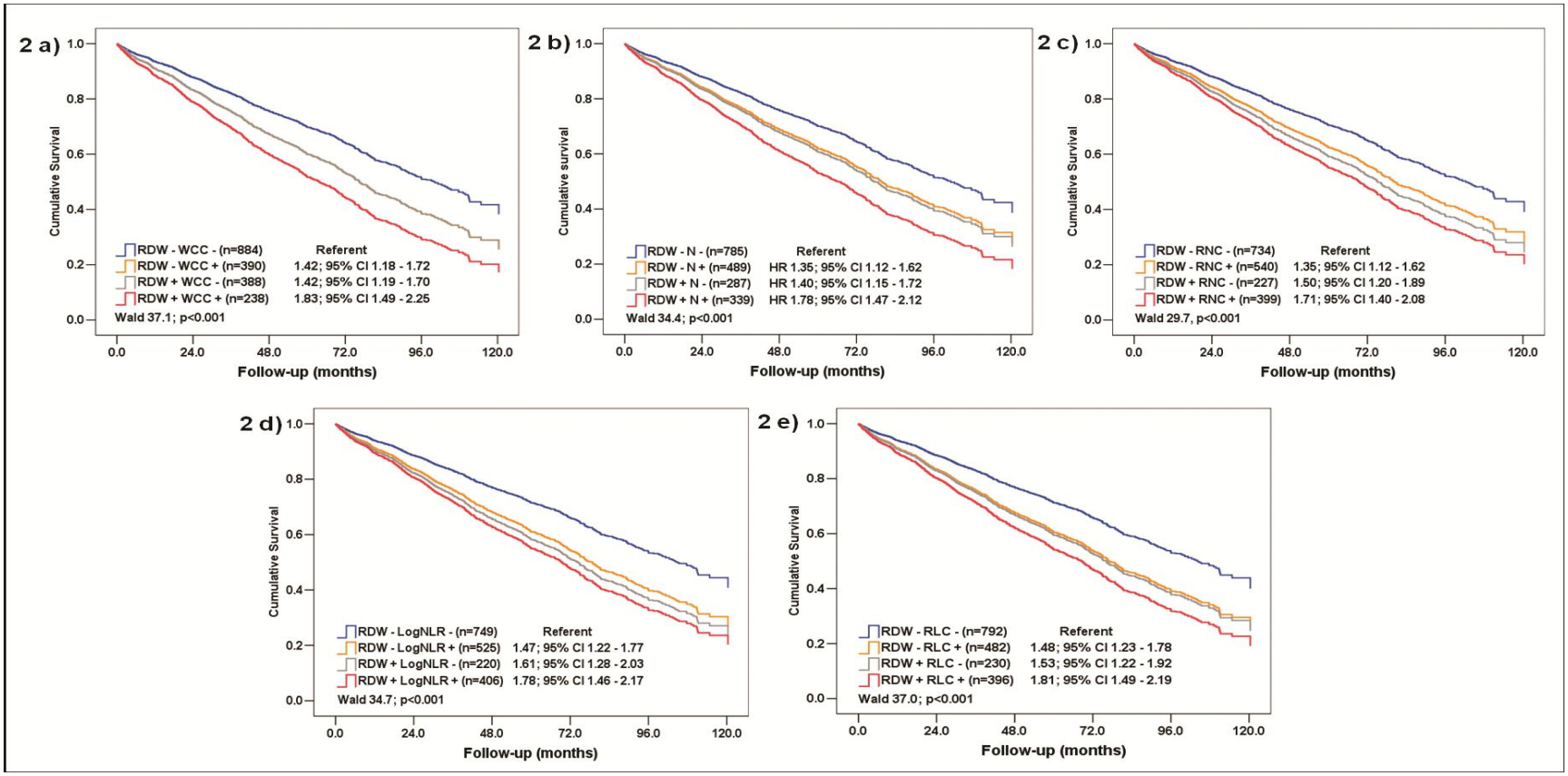


Figure 4.4.2 Adjusted survival curves in patients divided into subgroups according to the thresholds derived from ROC curves with RDW < 14.9% (RDW -) or RDW ≥ 14.9% (RDW +) and a) WCC < 7.95 x 10^9/L (WCC -) or ≥ 7.95 x 10^9/L (WCC +); b) neutrophil count < 4.79 x 10^9/L (N -) or ≥ 4.79 x 10^9/L (N +); c) relative neutrophil count < 64.8% (RNC -) or ≥ 64.8% (RNC +); d) relative lymphocyte count > 22.1% (RLC -) or ≤ 22.1% (RLC +); and e) log[NLR] < 0.46 (log[NLR] -) or ≥ 0.46 (log[NLR] +).

Table 4.4.1 Baseline characteristics of all patients (n=1900) and those who had completed at least 1-year follow-up (n=1806)

	All patients	One-year survival		
	n=1900	Alive n=1609	Death n=197	*p*†
Age (years)	72.0 (64.2 – 78.0)	71.5 (63.6 – 77.5)	75.2 (68.5 – 79.9)	<0.001
Male (%)	73.4	73.8	69.6	0.205
BMI (kg/m)	27.6 (24.5 – 31.4)	27.7 (24.7 – 31.4)	25.8 (22.5 – 29.4)	<0.001
NHYA (%) I/II	67.0	69.9	49.7	<0.001
III/IV	33.0	30.1	50.3	
LVSD (%) I	26.1	26.5	21.3	<0.001
II	42.3	43.6	32.0	
III	31.6	29.9	46.7	
IHD (%)	69.4	69.7	72.6	0.399
Diabetes mellitus (%)	23.9	24.1	21.8	0.489
Atrial arrhythmia (%)	33.8	32.8	40.6	0.029
Hypertension (%)	33.8	35.2	26.9	0.021
Non-cardiac Vascular disease (%)	16.4	16.5	22.3	0.039
Medications (%)				
ACEI/ARB	79.1	79.4	75.1	0.169
Beta-blockers	58.4	41.0	46.2	0.001
Diuretics	74.3	73.5	81.2	0.020
ARA	24.4	23.6	23.9	0.925
Clinical				
Heart rate (bpm)	72 (62 – 84)	71 (61 – 84)	80 (67 – 89)	<0.001
SBP (mmHg)	130 (115 – 148)	131 (116 – 148)	120 (103 – 140)	<0.001
DBP (mmHg)	76 (67 – 85)	77 (68 – 86)	71 (63 – 80)	<0.001
Sodium (mmol/L)	139 (137 – 141)	139 (137 – 141)	137 (135 – 140)	<0.001
Urea (mmol/L)	6.7 (5.1 – 9.2)	6.6 (5.1 – 8.9)	7.8 (5.9 – 11.3)	<0.001
Creatinine (μmol/L)	104 (87 – 127)	104 (87 – 126)	114 (90 – 139)	0.004
GFR	61.5 ± 21.1	61.8 ± 20.5	57.2 ± 23.7	0.001

(ml/min/1.73m^2)				
Hemoglobin (g/dL)	13.5 (12.3 – 14.6)	13.6 (12.4 – 14.6)	12.6 (11.5 – 13.9)	<0.001
RDW (%)	14.2 (13.5 – 15.3)	14.1 (11.7 – 15.2)	15.0 (13.9 – 16.7)	<0.001
WCC (x10^9/L)	7.2 (6.0 – 8.5)	7.1 (5.9 – 8.3)	7.6 (6.1 – 9.0)	0.004
Neutrophils (x10^9/L)	4.5 (3.7 – 5.6)	4.4 (3.6 – 5.5)	5.1 (4.0 – 6.6)	<0.001
Lymphocytes (x10^9/L)	1.6 (1.2 – 2.1)	1.7 (1.2 – 2.1)	1.4 (1.1 – 1.8)	<0.001
NLR	2.8 (2.0 – 3.9)	2.7 (2.0 – 3.8)	3.5 (2.7 – 5.5)	<0.001
Log(NLR)	0.45 (0.30 – 0.59)	0.43 (0.30 – 0.57)	0.54 (0.44 – 0.74)	<0.001
RNC (%)	64.6 (58.1 – 70.0)	64.1 (57.5 – 69.6)	68.2 (63.5 – 75.1)	<0.001
RLC (%)	22.8 (17.7 – 28.8)	23.5 (18.3 – 29.3)	18.9 (13.7 – 23.3)	<0.001
Platelets (x10^9/L)	222 (183 – 267)	221 (183 – 264)	234 (180 – 289)	0.099
NT-proBNP (pmol/L)	1346 (547 – 3560)	1193 (498 – 2975)	4059 (1682 – 7750)	<0.001
Log(NT-proBNP)	3.13 (2.74 – 3.55)	3.08 (2.70 – 3.47)	3.61 (3.23 – 3.89)	<0.001
hs-CRP (mg/L)‡	8.0 (4.0 – 17.0)	7.0 (3.0 – 14.0)	12 (5.5 – 41.5)	<0.001

ARA, aldosterone receptor antagonist; ACEI/ARB, angiotensin converting enzyme inhibitor or angiotensin receptor blocker; BMI,body mass index; GFR, glomerular filtration rate; hs-CRP, high-sensitivity CRP; IHD, ischaemic heart disease; LVSD, left ventricular systolic dysfunction; NLR, neutrophil-to-lymphocyte ratio; NT-proBNP, amino terminal brain natriuretic peptide; NYHA, New York Heart Association functional class; RDW, red cell distribution width; RLC, relative lymphocyte count; RNC, relative neutrophil count; WCC, white cell count.

†Comparing patients who were alive to those who died by the end of the first year's follow-up. ‡n=327 (18.1%) with hs-CRP test available.

Table 4.4.2 Pearson correlations between the blood count variables with each other and with other clinical and laboratory variables

Variables	Hb	RDW	WCC	Neutrophils	Lymphocytes	RNC	RLC	Log(NLR)
Other variables:								
Age	-0.28*	0.15*	-0.06†	0.01	-0.20*	0.16*	-0.20*	0.19*
LVEF (n=1337)	-0.03	-0.16*	-0.01	-0.06†	0.06†	-0.09*	-0.09*	-0.10*
Systolic BP	0.10*	-0.11*	-0.03	-0.04	0.04	-0.05†	0.08*	-0.07*
Diastolic BP	0.30*	-0.11*	-0.02	-0.04	0.08*	0.07*	0.09*	-0.09*
Sodium	0.13*	-0.05†	-0.06†	-0.09*	0.10*	-0.10*	0.15*	-0.14*
Urea	-0.30*	0.22*	0.09*	0.15*	-0.14*	0.19*	-0.22*	0.22*
Creatinine	-0.17*	0.17*	0.04	0.10*	-0.16*	0.16*	-0.21*	0.20*
GFR	0.30*	-0.22*	-0.04	-0.11*	0.16*	-0.17*	0.21*	-0.20*
Log(NT-proBNP)	-0.30*	0.39*	0.05†	0.18*	-0.31*	0.32*	-0.37*	0.37*
hs-CRP (n=327)	-0.30*	0.22*	0.33*	0.40*	-0.16*	0.38*	-0.36*	0.37*
Blood count variables:								
Hb	--	-0.34*	0.05*	-0.02	0.21*	-0.14*	0.20*	-0.20*
RDW	--	--	0.12*	0.18*	-0.14*	0.23*	-0.26*	0.27*
WCC	--	--	--	0.92*	0.44*	0.29*	-0.23*	0.27*
Neutrophils	--	--	--	--	0.07*	0.64*	-0.55*	0.61*
Lymphocytes	--	--	--	--	--	-0.62*	0.74*	-0.70*
RNC	--	--	--	--	--	--	-0.91*	0.95*
RLC	--	--	--	--	--	--	--	-0.97*

* $p<0.001$ and † $p<0.01$

GFR, glomerular filtration rate; Hb, haemoglobin; hs-CRP, high-sensitivity c-reactive protein; LVEF, left ventricular ejection fraction; NLR, neutrophil-to-lymphocyte ratio; NT-proBNP, N-terminal pro-brain natriuretic peptide; RDW, red cell distribution width; RLC, relative lymphocyte count; RNC; relative neutrophil count; BP, blood pressure; WCC, white cell count.

Table 4.4.3 The blood count variables in different subgroups of patients

		Hb (g/dL)	RDW (%)	WCC (x 10^9/L)	NEC (x 10^9/L)	LYC (x 10^9/L)	RNC (%)	RLC (%)	Log(NLR)	Log (NT-proBNP)
Sex	Male (n=1394)	13.9‡ (12.6-14.9)	14.1† (13.5-15.2)	7.1 (5.9-8.4)	4.5 (3.7-5.6)	1.6† (1.2-2.1)	64.5 (58.0-69.8)	22.5 (17.6-28.6)	0.46 (0.30-0.59)	3.1† (2.7-3.5)
	Female (n=506)	12.8 (11.8-13.7)	14.4 (13.5-15.5)	7.2 (6.0-8.7)	4.6 (3.7-5.9)	1.7 (1.2-2.2)	65.0 (58.4-7.4)	23.8 (18.1-29.4)	0.44 (0.30-0.58)	3.2 (2.8-3.6)
NYHA	I/II (n=1273)	13.7‡ (12.5-14.7)	14.0‡ (13.3-14.9)	7.0‡ (5.8-8.3)	4.3‡ (3.5-5.4)	1.7‡ (1.3-2.2)	63.3‡ (56.9-68.9)	24.2‡ (19.1-30.0)	0.42‡ (0.28-0.55)	3.1‡ (2.7-3.5)
	III/IV (n=627)	13.1 (11.9-14.3)	14.7 (13.8-15.9)	7.5 (6.2-8.9)	4.9 (4.0-6.1)	1.5 (1.1-2.0)	67.0 (60.8-72.6)	20.5 (15.4-25.9)	0.51 (0.37-0.67)	3.3 (2.9-3.7)
LVSD	I (n=496)	13.5 (12.4-14.5)	13.9‡ (13.3-14.8)	7.1 (5.9-8.5)	4.4 (3.5-5.5)	1.7† (1.3-2.2)	63.1‡ (57.0-68.9)	24.2‡ (18.8-30.0)	0.42‡ (0.28-0.56)	2.9‡ (2.4-3.3)
	II (n=803)	13.5 (12.1-14.6)	14.2 (13.5-15.3)	7.2 (6.0-8.5)	4.6 (3.6-5.7)	1.6 (1.2-2.1)	64.2 (58.1-69.9)	23.0 (18.0-28.7)	0.45 (0.30-0.58)	3.1 (2.7-3.5)
	III (n=601)	13.7 (12.4-14.7)	14.5 (13.6-15.7)	7.1 (6.0-8.3)	4.6 (3.7-5.6)	1.5 (1.2-2.0)	65.8 (59.5-71.1)	21.7 (16.6-27.8)	0.48 (0.33-0.62)	3.4 (3.0-3.7)
IHD	No (n=581)	13.7‡ (12.4-15.0)	14.3 (13.5-15.5)	7.1 (6.0-8.3)	4.6 (3.7-5.6)	1.6 (1.2-2.0)	65.1* (59.0-70.8)	22.5 (17.4-28.1)	0.46 (0.32-0.61)	3.2 (2..8-3.6)
	Yes (n=1319)	13.5 (12.2-14.5)	14.2 (13.5-15.2)	7.2 (6.0-8.5)	4.5 (3.6-5.6)	1.6 (1.2-2.1)	64.4 (57.5-69.7)	23.1 (17.9-29.2)	0.45 (0.30-0.59)	3.1 (2.7-3.5)
DM	No (n=1446)	13.7‡ (12.4-14.7)	14.1† (13.4-15.2)	7.1† (5.9-8.4)	4.4‡ (3.6-5.6)	1.6* (1.2-2.1)	64.1* (57.7-69.8)	23.0 (17.9-28.8)	0.45 (0.30-0.59)	3.1 (2.7-3.6)
	Yes (n=454)	13.1 (12.0-14.4)	14.4 (13.6-15.5)	7.4 (6.2-8.8)	4.8 (3.9-5.8)	1.7 (1.2-2.2)	65.4 (59.2-70.5)	22.5 (17.3-29.0)	0.46 (0.30-0.61)	3.1 (2.7-3.6)
HBP	No (n=1257)	13.6 (12.3-14.6)	14.2 (13.4-15.3)	7.2 (6.0-8.5)	4.5 (3.7-5.6)	1.6 (1.2-2.1)	64.7 (58.1-69.8)	22.6 (17.7-28.9)	0.46 (0.30-0.59)	3.1 (2.7-3.6)
	Yes	13.5	14.2	7.1	4.5	1.6	64.5	23.2	0.44	3.1

	(n=643)	(12.3-14.5)	(13.5-15.3)	(5.9-8.4)	(3.6-5.6)	(1.2-2.1)	(58.2-70.3)	(17.8-28.7)	(0.31-0.59)	(2.7-3.5)
		Hb (g/dL)	RDW (%)	WCC (x 10^9/L)	NEC (x 10^9/L)	LYC (x 10^9/L)	RNC (%)	RLC (%)	Log(NLR)	Log (NT-proBNP)
AF	No (n=1257)	13.5 (12.2-14.5)	14.0‡ (13.4-15.1)	7.1 (5.9-8.5)	4.4† (3.6-5.6)	1.7‡ (1.3-2.2)	63.5‡ (57.1-69.5)	23.9‡ (18.4-29.6)	0.42‡ (0.29-0.57)	3.0‡ (2.6-3.5)
	Yes (n=643)	13.6 (12.4-14.7)	14.5 (13.8-15.7)	7.2 (6.0-8.4)	4.7 (3.7-5.7)	1.5 (1.2-1.9)	65.9 (60.4-70.9)	20.8 (17.0-27.0)	0.50 (0.35-0.62)	3.3 (3.0-3.6)
NCVD	No (n=1582)	13.6† (12.4-14.6)	14.1‡ (13.4-15.2)	7.1† (5.9-8.4)	4.4† (3.6-5.6)	1.6 (1.2-2.1)	64.4 (58.0-70.0)	23.0 (17.9-28.9)	0.44 (0.30-0.59)	3.1† (2.7-3.5)
	Yes (n=318)	13.1 (11.9-14.4)	14.5 (13.7-15.7)	7.5 (6.2-8.8)	4.8 (3.9-6.0)	1.6 (1.2-2.1)	65.6 (59.3-70.6)	21.9 (17.1-28.2)	0.47 (0.33-0.62)	3.2 (2.9-3.6)
ACEI/ARB	No (n=397)	13.4 (12.2-14.5)	14.3 (13.5-15.5)	7.1 (6.0-8.5)	4.7 (3.7-5.8)	1.5† (1.2-2.0)	65.7† (59.5-71.2)	21.7† (16.9-27.5)	0.48† (0.34-0.62)	3.2‡ (2.8-3.7)
	Yes (n=1503)	13.6 (12.3-14.6)	14.2 (13.5-15.3)	7.2 (6.0-8.5)	4.5 (3.7-5.6)	1.7 (1.2-2.1)	64.2 (57.8-69.7)	23.4 (18.0-29.2)	0.44 (0.30-0.58)	3.1 (2.7-3.5)
BB	No (n=791)	13.5 (12.2-14.5)	14.5‡ (13.7-15.6)	7.2 (5.9-8.5)	4.7† (3.8-5.9)	1.5‡ (1.1-2.0)	66.0‡ (60.0-71.5)	21.6‡ (16.5-27.4)	0.48‡ (0.34-0.63)	3.2‡ (2.8-3.6)
	Yes (n=1109)	13.6 (12.4-14.7)	14.0 (13.4-15.0)	7.2 (6.0-8.5)	4.4 (3.7-5.5)	1.7 (1.2-2.2)	63.4 (57.0-69.3)	23.9 (18.6-30.0)	0.42 (0.28-0.57)	3.1 (2.7-3.5)
Diuretics	No (n=489)	14.0‡ (13.0-15.0)	13.8‡ (13.2-14.5)	7.0† (5.8-8.2)	4.3‡ (3.4-5.3)	1.7‡ (1.3-2.2)	62.3‡ (55.9-67.8)	25.2‡ (20.3-30.6)	0.39‡ (0.27-0.52)	2.9‡ (2.4-3.3)
	Yes (n=1411)	13.3 (12.1-14.4)	14.4 (13.6-15.6)	7.2 (6.1-8.5)	4.6 (3.7-5.7)	1.6 (1.2-2.1)	65.2 (59.0-70.6)	22.0 (17.1-28.0)	0.47 (0.33-0.61)	3.2 (2.9-3.6)

Data are presented as median (inter-quartile range)
* $p<0.05$; † $p<0.01$; and ‡ $p<0.001$
ACEI/ARB, angiotensin converting enzyme inhibitor or angiotensin receptor blocker; AF, atrial arrhythmia; BB, beta-blocker; DM, diabetes mellitus; HBP, hypertension; IHD, ischaemic heart disease; LVSD, left ventricular systolic dysfunction; NCVD, non-cardiac vascular disease; NYHA, New York Heart Association breathlessness classification;

Table 4.4.4 The Youden indices, area under the curve (AUC), sensitivity and specificity for the white and red cell variables in predicting 1-year mortality

	Youden index	AUC	Sensitivity (%)	Specificity (%)
Haemoglobin (g/dL)	13.3	0.74 (0.70-0.78)	58	65
RDW (%)	14.9	0.64 (0.60-0.68)	55	70
WCC ($x10^9$/L)	7.95	0.56 (0.52-0.61)	45	69
Neutrophils ($x10^9$/L)	4.79	0.62 (0.57-0.66)	60	59
Lymphocytes ($x10^9$/L)	1.72	0.63 (0.62-0.70)	46	74
RNC (%)	64.8	0.66 (0.62-0.70)	72	53
RLC (%)	22.1	0.67 (0.63-0.71)	57	53
log[NLR]	0.46	0.67 (0.63-0.71)	73	54
Log[NT-proBNP]	3.35	0.74 (0.70-0.78)	72	68

NLR, neutrophil-to-lymphocyte ratio; RDW, red cell distribution width; RLC, relative lymphocyte count; rNC, relative neutrophil count; WCC, white cell count

Table 4.4.5 Uni-variable and forward stepwise Cox regression modelling to identify the predictors of long-term mortality prior to the inclusion of white cell variables (n=1900)

	Uni-variable			Multi-variable*		
	Wald	HR (95% CI)	*p*	Wald	HR (95% CI)	*p*
Age (years)	215.0	1.06 (1.05 – 1.07)	<0.001	82.4	1.04 (1.03 – 1.05)	<0.001
Men	4.4	0.86 (0.74 – 0.99)	0.036		--	
BMI (kg/m^2)	26.1	0.97 (0.96 – 0.98)	<0.001		--	
NYHA III/IV	92.0	1.92 (1.68 – 2.20)	<0.001	14.9	1.33 (1.15 – 1.53)	<0.001
LVSD	18.9		<0.001			
Mild-moderate		1.00			--	
Moderate		1.09 (0.92 – 1.30)			--	
Severe		1.42 (1.19 – 1.70)			--	
IHD	11.0	1.29 (1.11 – 1.50)	0.001	8.4	1.26 (1.08 – 1.48)	0.004
Atrial arrhythmia	21.6	1.38 (1.20 – 1.58)	<0.001		--	
Other vascular disease	29.7	1.55 (1.32 – 1.82)	<0.001	6.7	1.24 (1.05 – 1.46)	0.01
ACEI or ARB	4.9	0.84 (0.72 – 0.98)	0.027		--	
β-blockers	26.7	0.71 (0.62 – 0.81)	<0.001		--	
Loop diuretics	52.1	1.88 (1.58 – 2.32)	<0.001		--	
Heart rate (bpm)	11.0	1.01 (1.00 – 1.01)	0.001		--	
Systolic BP (mmHg)	7.3	0.99 (0.99 – 1.00)	0.007		--	
Diastolic BP (mmHg)	54.9	0.98 (0.98 – 0.99)	<0.001	7.5	0.99 (0.99 – 1.00)	0.006
Sodium (mmol/L)	44.0	0.94 (0.93 – 0.96)	<0.001	10.1	0.97 (0.95 – 0.99)	0.002
Urea (mmol/L)	174.0	1.06 (1.05 – 1.07)	<0.001	16.8	1.03 (1.01 – 1.04)	<0.001
GFR ($ml/min/1.73m^2$)	135.2	0.98 (0.97 – 0.98)	<0.001		--	
log(NT-proBNP)	304.4	3.03 (2.67 – 3.42)	<0.001	85.2	1.96 (1.70 – 2.27)	<0.001
Hb (g/dL)	119.1	0.81 (0.78 – 0.84)	<0.001		--	
RDW (%)	178.5	1.22 (1.19 – 1.26)	<0.001	24.4	1.10 (1.06 – 1.14)	<0.001
WCC ($x\ 10^9/L$)	20.4	1.08 (1.04 – 1.11)	<0.001	13.2	1.06 (1.03 – 1.10)	<0.001

Neutrophil ($x\ 10^9$/L)	60.9	1.15 (1.11 – 1.19)	<0.001	21.0	1.10 (1.05 – 1.14)	<0.001
Lymphocyte ($x\ 10^9$/L)	66.2	0.63 (0.59 – 0.70)	<0.001	2.2	0.92 (0.83 – 1.03)	0.14
RNC (%)	110.0	1.04 (1.03 – 1.05)	<0.001	15.4	1.02 (1.01 – 1.03)	<0.001
RLC (%)	158.5	0.94 (0.94 – 0.95)	<0.003	19.7	0.98 (0.97 – 0.99)	<0.001
Log[NLR]	167.9	6.86 (5.13 – 9.18)	<0.001	19.7	2.09 (1.51 – 2.90)	<0.001

*All except the white cell variables were included in the forward stepwise Cox multi-variable model. The χ^2 of this model was 546.

†Each of the white cell variables was entered separately into the Cox multi-variable model. The χ^2 of model following the addition of white cell count, neutrophil, lymphocyte, relative neutrophil, relative lymphocyte and log[NLR] were 564, 570, 559, 567, 572 and 576 respectively.

ACEI, angiotensin converting enzyme inhibitors; ARB, angiotension receptor blocker; BP, blood pressure; GFR, glomerular filtration rate; IHD, ischaemic heart disease; LVSD, left ventricular systolic dysfunction; NLR, neutrophil-to-lymphoctye ratio; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Failure Association Classification; RDW, red cell distribution width; RLC, relative lymphocyte count; RNC, relative neutrophil count; WCC, white cell count; χ^2, Chi square.

Table 4.4.6 The c-statistic of Cox models following the addition of log[NT-proBNP], RDW and the white cell variables sequentially or in combination

Models	c-statistcis
Base model*	0.686
Base model + log[NT-proBNP]	0.716
Base model + log[NT-proBNP] + RDW	0.722
Base model + log[NT-proBNP] + WCC	0.720
Base model + log[NT-proBNP] + neutrophil	0.722
Base model + log[NT-proBNP] + relative neutrophil	0.720
Base model + log[NT-proBNP] + relative lymphocyte	0.722
Base model + log[NT-proBNP] + log[NLR]	0.722
Base model + log[NT-proBNP] + RDW + WCC	0.725
Base model + log[NT-proBNP] + RDW + neutrophil	0.726
Base model + log[NT-proBNP] + RDW + relative neutrophil	0.724
Base model + log[NT-proBNP] + RDW + relative lymphocyte	0.726
Base model + log[NT-proBNP] + RDW + log[NLR]	0.726
*Base model included age, diastolic blood pressure, urea, NYHA, presence of ischaemic heart disease, sodium and presence of other vascular disease. NLR, neutrophil-to-lymphocyte ratio; NT-proBNP, N-terminal B-type natriuretic peptide; RDW, red cell distribution width; WCC, white cell count.	

4.5 Discussion

To our knowledge, our study is the first to investigate the value of red and white cell variables in addition to NT-proBNP for stratifying the risk of patients with CHF. Information obtained from a routine haematology laboratory profile provided prognostic information independent of conventional variables including NT-proBNP and identified two potential therapeutic targets; anaemia and disturbed immune function.

Hb is a widely accepted prognostic marker.[391] RDW was a slightly stronger predictor of prognosis than Hb but if RDW was excluded from the model Hb replaced it, although there was only modest correlation between the two.[7] As mentioned earlier, the causes of anaemia in CHF are multi-factorial and include deficiency in iron and other haematinic agents,[392] renal dysfunction[393] and inflammation.[77,394] RDW reflects production of red cells with a greater variety of volumes and may have similar etiologies as anaemia itself.[124] However, there is one fundamental difference. Hb is measured as a concentration and, unlike RDW, will be influenced by variation in plasma volume that may cause dilutional anaemia. Recently, a large study of an agent, darbopoetin, used to stimulate bone marrow production of red cells failed to show that it could improve prognosis (RED-HF).[395] Its impact on RDW is unknown. On the other hand, there have been promising results with iron supplementation.[396]

Most studies that have investigated the prognostic value of white cell variables in heart failure involved only patients with decompensated heart failure[141,142] and/or did not include natriuretic peptides[143-147] or red cell variables[148,149]. In a retrospective analysis of the 6,642 patients in Studies of Left Ventricular Dysfunction (SOLVD),[148] increasing WCC and neutrophils but not lymphocytes were independent predictors of long-term all-cause and cardiovascular mortality in patients with CHF on stable medications. However, natriuretic peptides and red cell variables were not included in the analysis.

A retrospective analysis of the 5010 patients in Valsartan Heart Failure Trial (Val-HeFT) showed that increasing neutrophils and decreasing lymphocytes were independent predictors of death even with the inclusion BNP.[149] However, red cell variables were not included in the model. A lower RLC was identified as independent predictor of a worse prognosis in the Seattle Heart Failure Model.[150] However, the Seattle model does not include natriuretic peptides.

Compared to normal subjects, patients with CHF have a similar absolute white cell count but tend to have higher neutrophil and lower lymphocyte counts regardless of the aetiology of heart failure.[151,152] These differences are more pronounced in patients with severe CHF[143,148] or in those who are not taking a β-blocker.[151] It remains unclear if relative neutrophilia and lymphopenia is a mere consequence of the CHF syndrome or whether it plays a part in the progression of the disease. The neutrophils in patients with CHF are activated[153] and have increased lifespan due to a reduction in apoptosis.[154] On the other hand, increased sensitivity to cytokine-induced apoptosis and redistribution from peripheral blood to other sites have been postulated as the key mechanisms for relative lymphopenia.[155] Although the main lymphocyte subsets affected by heart failure appear to be the T helper and B cells, the activity of cytotoxic T cells,[151] natural killer cells[156] and T-suppressor cells[157] is also reduced in patients with CHF.

CHF is associated with a chronic inflammatory response[158] which may modulate the distribution of leukocytes. In common with other studies,[144,151] we found moderate correlations between white cell variables and hs-CRP. CHF is also associated with sympathetic activation. Chronic β-adrenergic receptor stimulation may increase neutrophil[161] and inhibit lymphocyte proliferation[159,160].

Activated neutrophils release a wide range of proteolytic enzymes such as myeloperoxidase which is associated with abnormal myocardial remodeling.[162] Relative lymphopenia may increase the predisposition to infection which is a common precipitating factor for decompensated heart failure and a cause of death in patients with CHF.[163] Although clinical trials of anti-cytokine therapy has been neutral in CHF,[164,165] more complex immunomodulation therapy targeted at preventing neutrophil activation and lymphocyte apoptosis remains to be investigated.

4.5.1 Limitations.

This is a single centre observational study and may be subject to bias and other confounding factors. Although FBC variables were associated with prognosis, the study was not designed to establish a causal relationship between these variables and mortality. Further, the specific cause of death was not available and only a small subgroup of patients had concomitant hs-CRP measurements. Nevertheless, we included consecutive patients referred to a heart failure clinic which reflects a ‘real-life’ clinical setting. Further studies are necessary to confirm our findings and establish if there is any causal relation between any of the variables and the progression of heart failure.

4.6 Conclusion

In patients with CHF, red and white cell indices obtained from the routine haematology profile provides additional prognostic information to other widely accepted prognostic variables, including NT-proBNP, at no additional cost.

Chapter 5 H-type fatty acid-binding protein in chronic heart failure

Heart-type fatty acid-binding protein in patients with chronic heart failure due to left ventricular systolic dysfunction

Some of the preliminary and short-term follow-up data have been presented in the British Cardiovascular Socitey Annual Scientific Conference.[397]

5.1 The incremental prognostic value of plasma heart-type fatty acid-binding protein (H-FABP) in patients with stable chronic heart failure due to left ventricular systolic dysfunction

5.1.1 Introduction

Progressive unfavourable left ventricular (LV) remodelling is associated with deterioration in LV function and a less favourable clinical outcome.[398,399] One key pathophysiological process involved in LV remodelling is ongoing myocardial damage which can occur even in stable ambulatory patients who are on optimal treatment.[400] That myocardial damage is ongoing is suggested by the finding of raised cardiac troponins (I or T: TnI or TnT) in approximately 11 – 25% of stable ambulatory patients[400] and up to 50% of patients being assessed for heart transplant.[17] In patients with chronic heart failure (CHF), raised cardiac troponins confers a worse prognosis with an incremental prognostic value in addition to brain natriuretic peptides (BNP).

As mentioned in Chapter 1.6, Heart-type fatty acid-binding protein (H-FABP) is a low molecular weight (15 kDa) soluble protein abundantly present in the cytoplasma of cardiomyocyte.[182] It facilitates intracellular long-chain fatty acid transport for oxidation and energy production,[179] protects cardiomyocytes against the potential adverse effects of locally high concentration of long-chain fatty acid from various sources[19] and regulates gene expression.[180]

H-FABP is released rapidly from cardiomyocytes during myocardial injury even in the absence of myofibril damage or necrosis and its level returns to baseline quickily, for example, within 20 hours following myocardial infarction.[188] This suggests that H-FABP may be a more sensitive and dynamic marker of ongoing myocardial damage than troponins.[22]

H-FABP is renally excreted and so may persist longer in patients with renal impairment.[187] In the absence of substantial renal impairment, H-FABP has incremental prognostic value in addition to BNP in patients with decompensated heart failure.[26-28,30,31] However, renal impairment is extremely common in patients with CHF[401] and so we investigated the prevalence of raised plasma H-FABP in unselected patients with stable CHF due to LVSD and the factors associated with raised H-FABP. We also examined its potential value in addition to NT-proBNP in predicting outcome.

5.1.2 Methods

5.1.2.1 Patients

Four hundred and eighty three consecutive patients with LVEF $\leq$ 40% managed by a heart failure clinic serving a local community who, were on stable heart failure treatment for at least 3 months before study entry were included (CHF group). For comparison, a group of 83 age- and sex-matched patients who had, or were at risk of developing, cardiovascular disease but in whom LVSD had been excluded were recruited (CVD group) together with 25 age-and sex-matched healthy volunteers (Normal group). Patients who had had acute coronary syndrome or decompensated HF within the preceding 4 weeks were excluded. The study was approved by Hull and East Riding Local Research Ethics Committee and the Research Board of Hull and East Yorkshire Hospitals NHS Trust. All the participants gave written informed consent.

5.1.2.2 Investigations

All patients were assessed and underwent investigation as described in Chapter 2.2.2 and 2.2.3. Additional blood samples were collected, stored and sent in batches to the core laboratory in McMaster University, Hamilton, Ontario, Canada for assay as described in Chapter 2.2.3. NT-proBNP was assayed in EDTA plasma (Elecsys 1010 analyser, Roche Diagnostics, Mannheim, Germany) whilst H-FABP was assayed in the citrated plasma (HyCult Biotechnology, Uden, The Netherlands).

5.1.2.3 Outcome measures

The primary outcome was the composite of heart failure hospitalisation and all-cause mortality. The secondary outcomes were HF hospitalisation, cardiovascular mortality and all-cause mortality.

5.1.2.4 Statistical analysis

Variables are presented and were analysed as described in Chapter 2.3. Raised H-FABP was defined as more than 3 standard deviations above the mean H-FABP of the Normal group. Univariable and multivariable binary logistic regression analyses were used to identify the factors associated with raised H-FABP. Their odd ratios (OR) and 95% confidence intervals (CI) are presented.

Pearson correlation between log[H-FABP] and log[NT-pro-BNP], age, BMI and GFR was performed. Based on the cut-off H-FABP level defined from the Normal subjects, Kaplan Meier survival curves were plotted. Receiver Operating Characteristic (ROC) curves were used to determine the area under the curves (AUC) for log[H-FABP] and log[NT-proBNP] in identifying those who reached the primary endpoint by 5-year follow-up. The AUC were compared using the methods described by Cleves.[364] Univariable Cox regression analysis was performed to identify the variables associated with prognosis as previously described in Chapter 2.3. As H-FABP level can be affected by age, sex, renal function and muscle mass or body mass index (BMI),[178,187,189] these factors were included in the multi-variable Cox model.

The incremental value of log[NT-proBNP] and log[H-FABP] in predicting the 5-year primary outcome was assessed by multiple methods as described in Chaper 2.3.[365] To estimate the prediction accuracy, c-statistic was calculated based the initial multivariable Cox model (the Base model). This was compared to the c-statistics of the models with the addition of log[NT-proBNP] and log[H-FABP] individually and both together.[366] The performance of each model and improvement of model performance were evaluated using calibration and IDI as decribed in Chapter 2.3.[367] The net NRI was calculated to evaluate the added predictive ability of log[NT-proBNP] and log[H-FABP].[367] For this, 4 clinically relevant groups, namely patients with the probabilities of suffering from a 5-year primary event of 1) < 10%, 2) 10 to < 20%, 3) 20 to 60% and 4) > 60%, were derived as described in Chapter 2.3. The patients were reclassified according to these risk groups for probabilities of a primary event at 5 years after the addition of log[NT-proBNP] and/or log[H-FABP] to the base model.

5.1.3 Results

5.1.3.1 Baseline comparison.

CHF patients had higher H-FABP and NT-proBNP but lower GFR and Hb levels than either CVD patients or Normal subjects. (Table 5.1.1)

The reference range derived from the Normal population for H-FABP was 1.46 ± 0.43ng/ml, giving an upper cut-off for raised H-FABP of 2.75ng/ml. (Figure 5.1.1) Thus, 7 (8.4%) CVD and 193 (40.0%) CHF patients had raised H-FABP. The H-FABP levels were similar in Normal subjects and CVD patients.

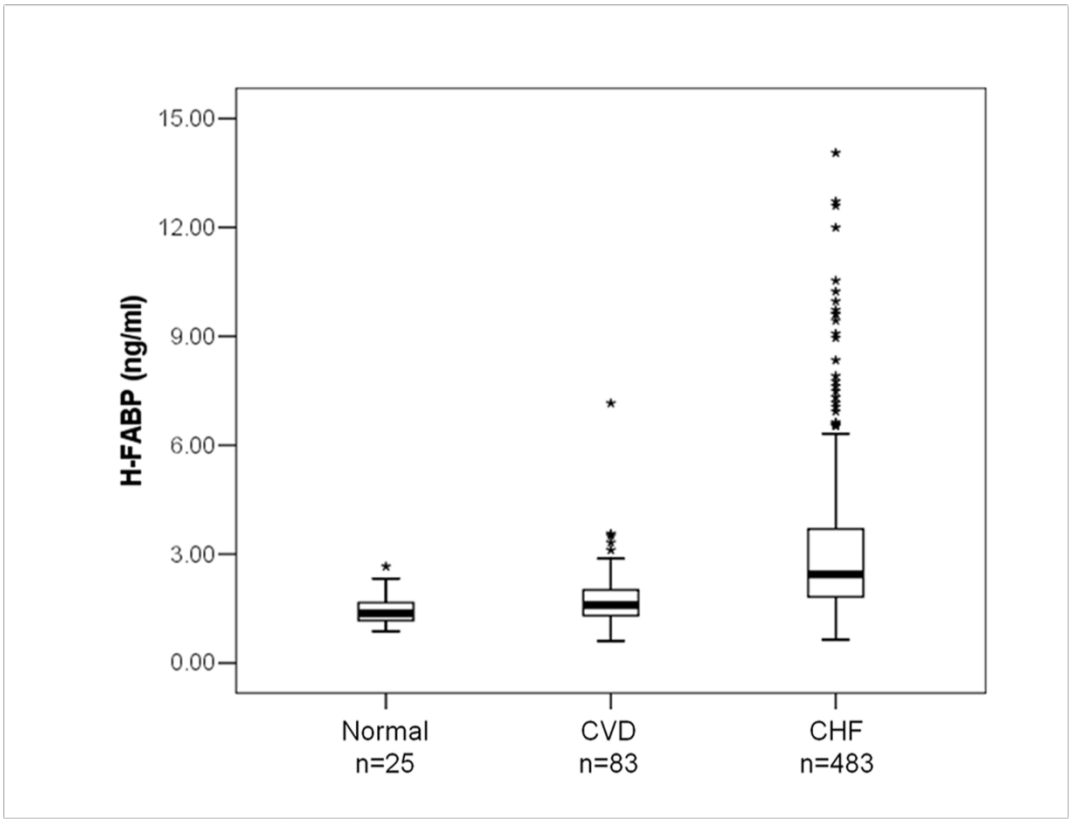


Figure 5.1.1 Box plot showing the districation of H-FABP in Normal subjects (n=25), CVD (n=83) and CHF (n=483) patients. Patients whose H-FABP levels were more than 2 standard deviations from the mean of each group are represented by an asterisk (*)

In CHF patients, the prevalence of raised H-FABP was 94.2% (49/52), 48.8% (120/246) and 13.0% (24/185) in those with GFR less than 30mls/min/1.73m^2, between 30 to 60mls/min/1.73m^2 and above 60mls/min/1.73m^2 respectively.

Of the factors associated with a raised H-FABP in all the subjects, only lower GFR and the presence of LVSD were the independent predictors. (Table 5.1.2) In CHF patients taken as a separate group, only GFR was an independent predictor of a raised H-FABP (OR 0.93; 95% CI 0.92 – 0.95, p<0.001).

In CHF patients alone, there were modest but significant correlations between log[H-FABP] and both age and log[NT-proBNP]. (Figure 5.1.2 a and b) There was a stronger inverse correlation between log[H-FABP] and GFR (Figure 5.1.2 c) but log[H-FABP] did not correlate with BMI. (Figure 5.1.2 d)

5.1.3.2 Prognosis and H-FABP

CHF patients were followed for a range of 68 - 90 (median 80; IQR 74-84) months. During follow-up, 238 (49.3%) patients died. One hundred and sixty eight deaths (70.6%) were due to a cardiovascular cause: 90 (53.6%) were due to progressive or end-stage heart failure, 54 (32.1%) were attributed to sudden cardiac death, 14 (8.3%) were due to a confirmed acute coronary event and 10 (6%) were due to other vascular causes. One hundred and sixty six (34.4%) of the patients had at least one hospital admission for decompensated HF. Overall, 291 (60.2%) patients died or experienced at least one episode of hospitalisation due to decompensated HF. Patients with raised H-FABP had higher combined HF hospitalisation and all-cause mortality, cardiovascular and all-cause mortality and HF hospitalisation as shown in the Kaplan Meier survival curves (Figure 5.1.3 a to d). The area under the ROC curve for log[H-FABP] and log[NT-proBNP] in identifying the combined end-point of 5-year heart failure hospitalisation and all-cause mortality were similar at 0.67 (95% confidence interval 0.62-0.72) and 0.70 (95% confidence interval 0.65-0.72) respectively, p=0.464. (Figure 5.1.4)

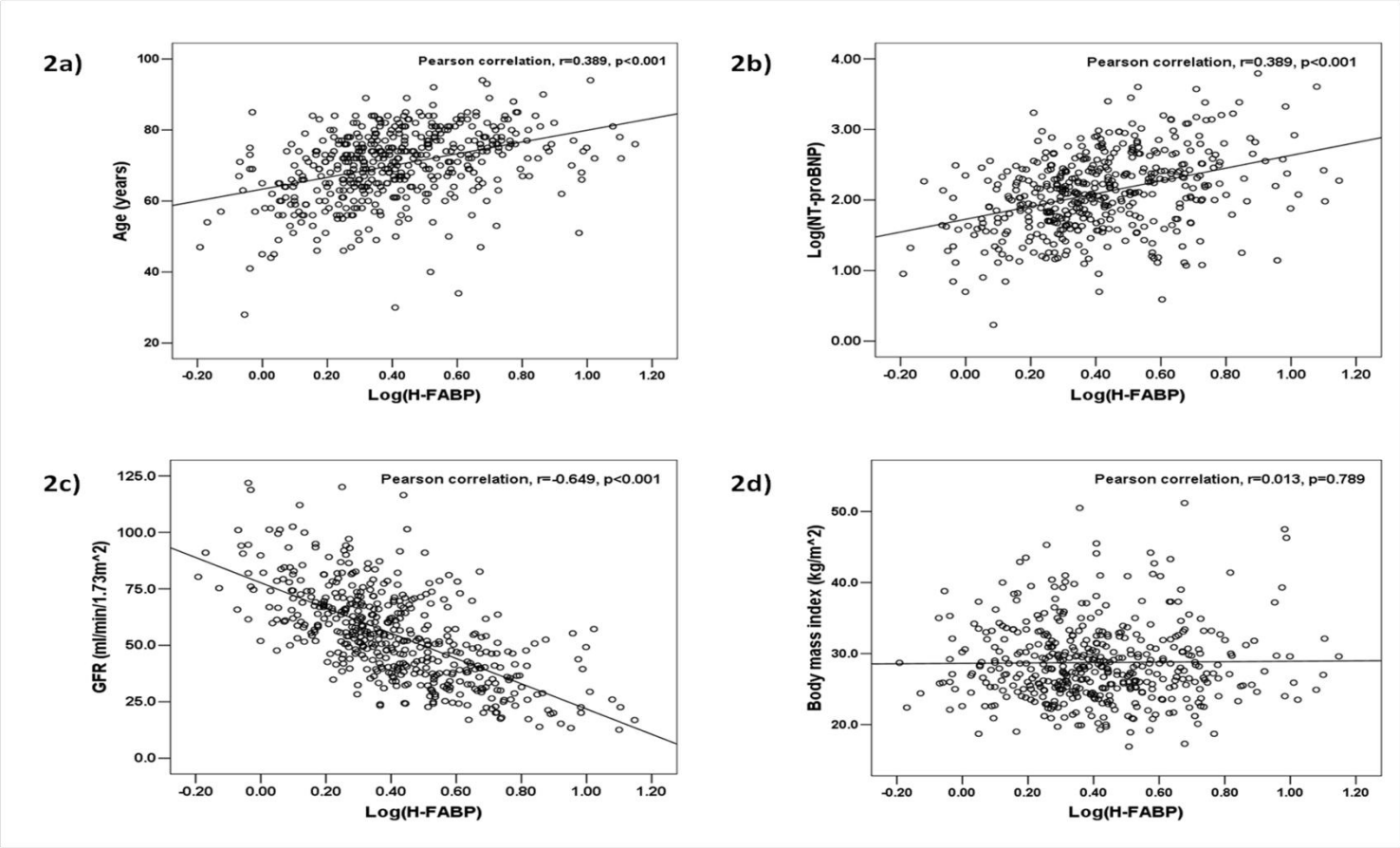


Figure 5.1.2 Log[H-FABP] correlated modestly with a) age and b)log[NT-proBNP] whilst having a stronger inverse correlation with c) GFR but did not correlate with d) BMI

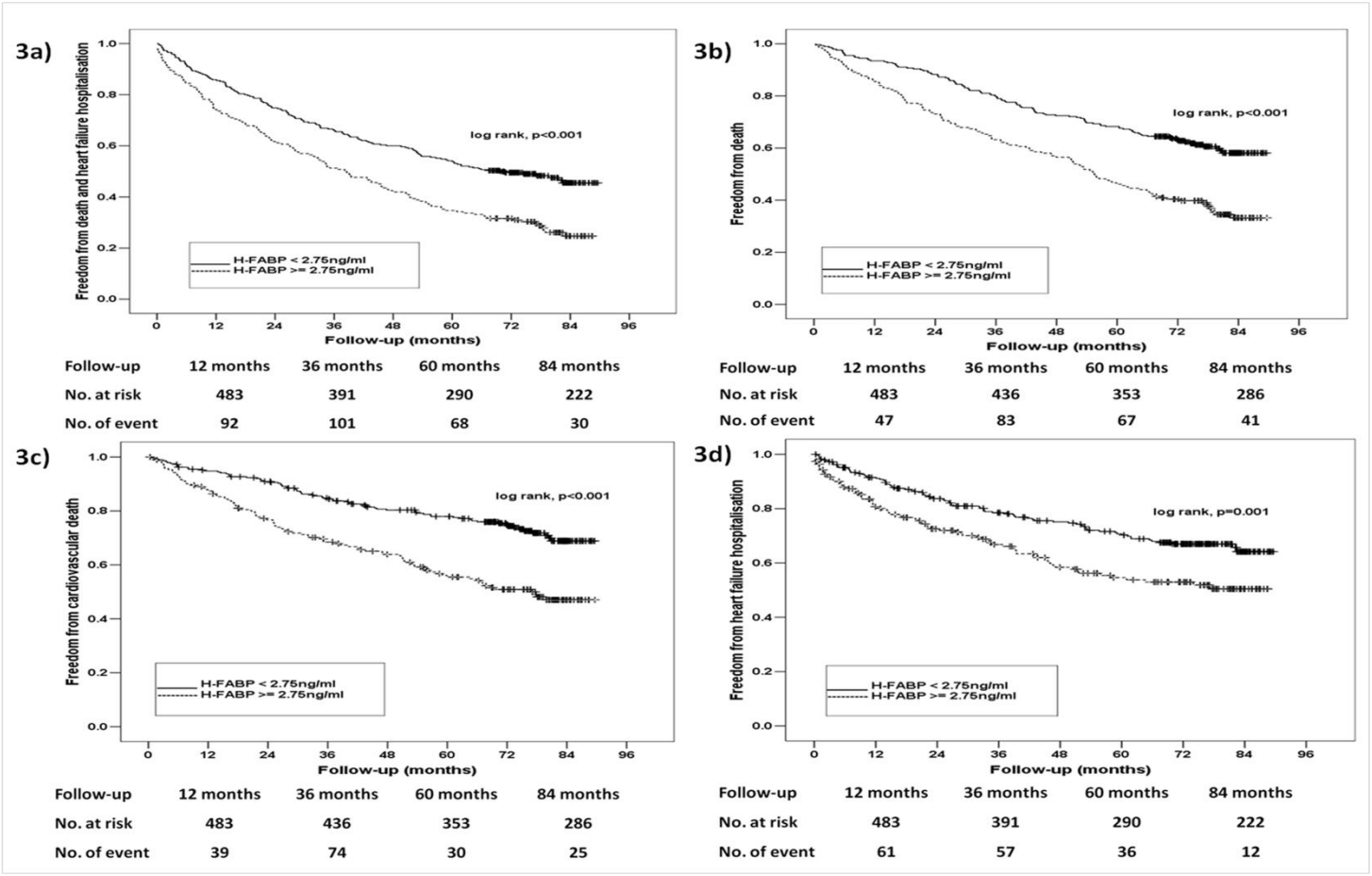


Figure 5.1.3 Kaplan Meier curves showing survival free from a) combined heart failure hospitalisation and all-cause mortality, b) all-cause mortality, c) cardiovascular mortality and d) heart failure hospitalisation in CHF patients with H-FABP < 2.75 ng/ml and ≥ 2.75ng/ml

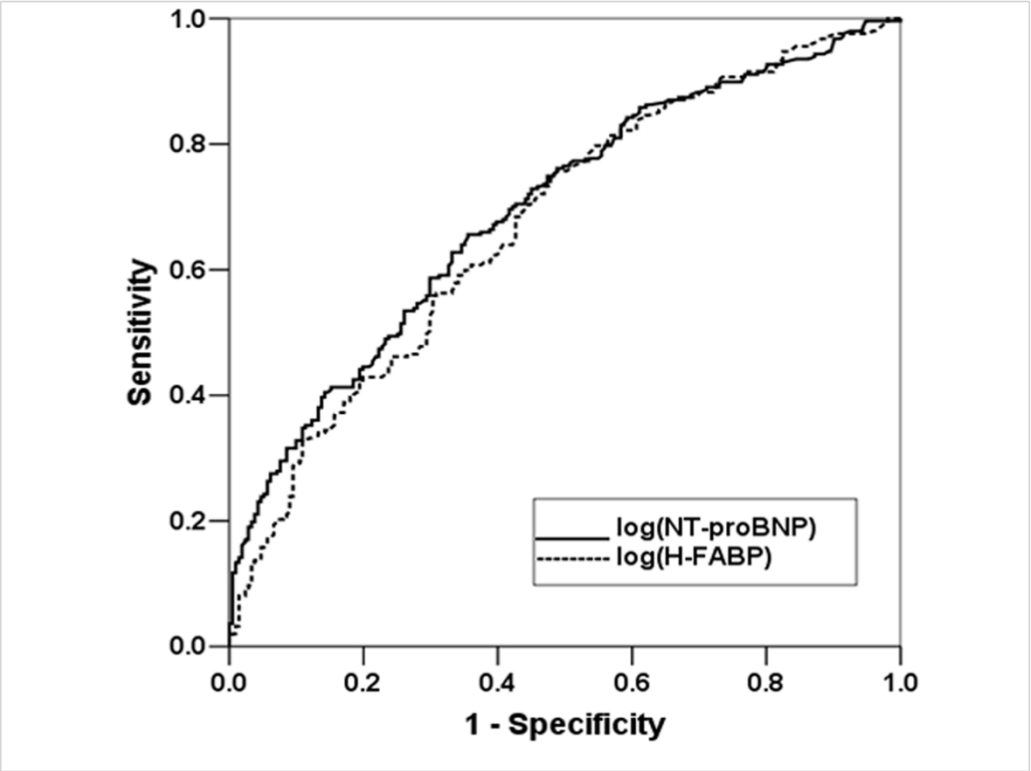


Figure 5.1.4 Receiver operator characteristic curves for log[NT-proBNP] and log[H-FABP] in relation to 5-year combined heart failure hospitalisation and all-cause mortality. The Youden point for log[H-FABP] was 0.3123 (H-FABP = 2.05 ng/ml) with a sensitivity of 75% and specificity of 50%; whilts that for log[NT-proBNP] was 2.088 (NTproBNP = 122.5 pmol/L) with a sensitivity of 66% and specificity of 64%

After adjustment for sex and BMI, higher log[H-FABP] and log[NT-proBNP], older age and more severe LVSD were independent predictors of a higher rate of the primary endpoint, whilst taking a β-blocker and higher Hb were associated with lower event rates. (Table 5.1.3) Log[H-FABP] was also an independent predictor of higher all-cause mortality (HR 3.06, 95% CI 1.30-7.21, p=0.011), cardiovascular mortality (HR 3.23; 95% CI 1.06-8.63, p=0.038) and hospitalisation for decompensated HF (HR 3.38; 95% CI 1.38-3.33, p=0.019).

5.1.3.3 Incremental value of H-FABP in risk stratification

The impact of adding log[NT-proBNP] and log[H-FABP] individually and together into the base model is shown in Table 5.1.4. Although the c-statistics did not

change when adding log[H-FABP] in addition to log[NT-proBNP] to the base model, the calibration of the model improved. The IDI also increased from 2% to 3.3% and NRI from 6.8% to 9.7% with the addition of log[H-FABP] to the base model + log[NT-proBNP]. (Table 5.1.4)

The Chi square for the multivariable Cox regression model (Table 5.1.3) without log[NT-proBNP] and log[H-FABP] was 143.2, $p<0.001$. This increased to 170.5, $p<0.001$ with the addition of log[NT-proBNP] to the model and 179.7, $p<0.001$ when both log[NT-proBNP] and log[H-FABP] were added.

Table 5.1.1 Baseline characteristics of normal volunteers (Normal), patients with established or at risk of developing cardiovascular disease (CVD) and patients with chronic heart failure (CHF)

	Normal n=25	CVD n=83	CHF n=483
Age (years)	71 (57-76)	68 (61-76)	72 (64-77)
Man	64	72.3	78.3
BMI (kg/m^2)	27.8 (26.1-29.6)	29.1 (26.3-32.4)	27.9 (25.1-31.8)
Diabetes mellitus		14.5	23.1
Hypertension		51.8	40.8
Family history	60.0	45.1	52.8†‖
Smoking history	60.0	75.9	75.4
IHD		62.7	77.4§
Other vascular disease		2.4	21.3‖
Atrial arrhythmia		3.6	30.9‖
Medications:			
Loop diuretics		27.7	81.4‖
ACEIs or ARBs		38.6	91.3‖
β-blockers		47.0	84.58‖
Statins		57.8	55.5
Anti-platelets		65.1	50.7‡
Warfarin		3.6	31.3‖
Laboratory tests:			
H-FABP (ng/ml)	1.4 (1.2-1.7)	1.6 (1.3-2.1)	2.5 (1.8-3.7)†‖
NT-proBNP (pmol/L)	8.1 (4.8-15.6)	14.6 (8.0-29.1)¶	127.4 (51.8-280.0)†‖
GFR ($ml/min/1.73m^2$)	78.8 (70.1-86.4)	73.4 (67.7-85.0)	53.6 (39.5-68.2)†‖
Hb (g/dL)	14.0 ± 1.5	14.0 ± 1.4	13.1 ± 1.5*‖
Sodium (mmol/L)	140 (138-141)	139 (138-141)	140 (138-141)
Albumin (g/L)	38(37-41)	39 (37-41)	38 (36-40)§

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; IHD, ischaemic heart disease.

Comparing CHF to Normal: * $p<0.05$ and † $p<0.001$; comparing CHF to CVD: ‡ $p<0.05$, § $p<0.01$ and ‖ $p<0.001$; comparing CVD to Normal: ¶ $p<0.05$.

Table 5.1.2 Factors associated with a raised H-FABP above 2.75 ng/ml in all subjects (n=591) and in patients with CHF (n=483)

	All subjects (n=591)			
	Univariable		Multi-variable	
	OR 95% CI	*p*	OR 95% CI	*p*
Age (years)	1.07 (1.05-1.10)	<0.001	1.02 (0.99-1.05)	0.113
Man	0.76 (0.50-1.15)	0.191		
BMI (kg/m^2)	0.99 (0.96-1.02)	0.445		
NYHA III/IV	2.36 (1.56-3.55)	<0.001	1.61 (0.93-2.78)	0.088
Angina	1.44 (0.97-2.14)	0.073		
Ischaemic heart disease	1.51 (1.02-2.24)	0.042	0.92 (0.55-1.55)	0.761
Atrial arrhythmias	1.70 (1.26-2.48)	0.007	1.10 (0.68-1.78)	0.707
Other vascular disease	1.68 (1.09-2.59)	0.018	0.65 (0.37-1.12)	0.122
LVSD		<0.001		0.042
None	1		1	
Mild-to-moderate	6.61 (2.80-15.56)		1.80 (0.64-5.07)	
Moderate	12.74 (5.65-28.72)		3.06 (1.11-8.44)	
Severe	8.57 (3.72-19.71)		1.74 (0.61-4.97)	
GFR ($mls/min/1.73m^2$)	0.92 (0.91-0.94)	<0.001	0.93 (0.92-0.95)	<0.001
Anemia	3.28 (2.3-4.67)	<0.001	1.53 (0.98-2.40)	0.061
Loop diuretics	2.93 (1.90-4.51)	<0.001	0.88 (0.48-1.62)	0.686
ACEI/ARBs	1.49 (0.95-2.35)	0.083		
β-blockers	1.62 (1.06-2.47)	0.025	0.83 (0.46-1.49)	0.552

Data are presented as odd ratio (OR) and 95% confidence interval (95% CI).
ARA, aldosterone receptor antagonist; NYHA, New York Heart Association classification.

Table 5.1.3 The predictors of combined heart failure hospitalisation and all-cause mortality in patients with CHF using Cox regression model

	Univariable		Multivariable	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (years)	1.05 (1.03-1.06)	<0.001	1.02 (1.00-1.04)	0.017
NYHA III/IV	1.95 (1.50-2.53)	<0.001	1.35 (1.00-1.83)	0.048
LVSD				
Mild-to-moderate	1.00	<0.001	1.00	0.001
Moderate	1.30 (0.95-1.80)		1.06 (0.76-1.49)	
Severe	1.90 (1.47-2.44)		1.78 (1.24-2.54)	
Ischemic heart disease	1.07 (0.80-1.42)	0.395		
Myocardial infarction	0.78 (0.61-1.00)	0.045	0.96 (0.73-1.26)	0.775
Atrial arrhythmias	1.41 (1.10-1.81)	0.007	1.07 (0.81-1.40)	0.647
Other vascular disease	1.58 (1.21-2.08)	0.001	1.35 (1.00-1.82)	0.054
Diabetes mellitus	1.38 (1.04-1.84)	0.026	1.18 (0.86-1.61)	0.308
ACEI or ARB	0.65 (0.45-0.96)	0.031	1.02 (0.67-1.57)	0.921
β-blockers	0.59 (0.44-0.80)	0.001	0.64 (0.46-0.88)	0.005
Loop diuretics	2.03 (1.43-2.88)	<0.001	1.30 (0.88-1.92)	0.184
Statins	0.76 (0.60-0.97)	0.026	0.97 (0.75-1.24)	0.792
log(H-FABP)	6.12 (3.73-10.06)	<0.001	2.75 (1.31-5.77)	0.007
log(NT-proBNP)	3.58 (2.75-4.64)	<0.001	2.31 (1.68-3.17)	<0.001
GFR (ml/min/1.73m^2)	0.98 (0.97-0.99)	<0.001	1.00 (0.99-1.01)	0.520
Hemoglobin (g/dL)	0.81 (0.75-0.87)	<0.001	0.91 (0.84-0.99)	0.031
Albumin (g/dL)	0.91 (0.87-0.95)	<0.001	1.00 (0.96-1.05)	0.911
Sodium (mmol/L)	0.98 (0.94-1.03)	0.114		

All variables adjusted for sex and BMI.

Table 5.1.4 The c-statistics, calibrations, integrateddiscrimination improvement (IDI) and net reclassification improvement (NRI) of the combined 5-year heart failure hospitalisation and all-cause mortality for the base model and after addition of log[NT-proBNP] and/or log[H-FABP]

Model	c-statistic*	Calibration	IDI*	NRI*†
Base model	0.767(0.724-0.810)	6.70 (p=0.569)		
Base model + log[NT-proBNP]	0.780 (0.737-0.822) (*p=0.127*)	6.70 (p=0.570)	0.020 (*p=0.002*)	6.8% (*p=0.038*)
Base model + log[H-FABP]	0.778 (0.736-0.819) (*p=0.201*)	3.62 (p=0.889)	0.019 (*p=0.005*)	8.4% (*p=0.019*)
Base model + log[NT-proBNP] + log[H-FABP]	0.786 (0.745-0.828) (*p=0.06*)	4.89 (p=0.770)	0.033 (*p<0.001*)	9.7% (*p=0.008*)

**p* represents comparison to Base model.

†Clinically relevant categories for NRI were probability of a 5-year combined HF hospitalisation and death of < 10%, 10% to < 20%, 20% to < 60% and $\geq$ 60%.

5.1.4 Discussion

We have studied H-FABP in the largest cohort of unselected stable CHF patients yet reported and have demonstrated for the first time, that a higher H-FABP is associated with a worse prognosis and has an incremental prognostic value over NT-proBNP and other established prognostic factors. We found that H-FABP was raised in 40% patients with HF due to LVSD and on stable medical therapy. Lower GFR and the presence of LVSD were independently associated with a raised H-FABP.

Others have found that a greater proportion of patients had a raised H-FABP.[23-31,402] However, all but one of these studies[402] involved patients with acute decompensated heart failure and the majority used cut-off levels to defined raised H-FABP that were derived from ROC analysis performed for prognostic purposes. The threshold we derived was lower than the 6ng/ml reference level suggested by other studies, but the 6ng/ml level was derived for the diagnosis of acute coronary syndrome.[403]

The mechanism(s) of troponin or H-FABP release in patients with stable CHF is unknown. One consistent finding is that troponin and H-FABP are detectable in CHF patients even in the absence of clinically overt myocardial ischaemia or obstructive coronary disease. However, increased left ventricular wall stress in LVSD can lead to an impairment of regional myocardial flow reserve without either significant coronary disease or increased myocardial oxygen consumption, hence subclinical ischaemia and abnormal myocyte metabolism can occur.[13] This may explains why H-FABP was not related to the presence of ischemic heart disease. In addition, myocardial remodelling may involve apoptosis and H-FABP correlates with blood soluble Fas molecules in patients with CHF, suggesting that the Fas/Fas ligand system (which can initiate apoptosis in cardiomyocytes)[10,14] is activated. Cardiomyocyte damage may also be due to activation of neuro-endocrine systems, inflammatory cytokines and oxidative stress.[10] Because H-FABP protects cardiomyocytes from high local concentrations of fatty acid induced by ischaemia,[19,179] the release of H-FABP from cardiomyocyte induced by myocardial injury might also lead to further deterioration in cardiomyocyte function, hence perpetuating a vicious cycle.

H-FABP clearance is predominantly through the kidneys[187] which explains the strong inverse correlation between GFR and log[H-FABP]: lower GFR was the only independent predictor of a raised H-FABP level in CHF patients. The clinical interpretation of H-FABP level has to be made in conjunction with renal function. The effect of renal clearance on H-FABP level may also partly explain the modest correlation between age and log[H-FABP] since renal function decreases with age.[19,189]

We did not find a relationship between either sex or body mass index and H-FABP as reported in previous studies.[19,189] Earlier studies found low concentrations of H-FABP in skeletal muscle which may explain the apparent relation between H-FABP and both sex and body mass. However, the finding may be due to cross-reactivity of earlier assays with other fatty acid-binding proteins (FABPs) which have 20% - 80% amino acid sequence homology with H-FABP. The newer assay used in our study has better sensitivity with less chance of cross-reactivity with other FABPs.[184]

Niizeki et al found that raised H-FABP was more common than raised TnT in patients with decompensated heart failure and suggested that H-FABP may be a more sensitive marker to detect ongoing myocardial damage.[22] However, patients with serum creatinine above 1.8 mg/dL (159μmol/L) were excluded from their study. Most studies

investigating the prognostic role of H-FABP have included only a small number of patients with decompensated heart failure and those without significant renal impairment; while some studies only focused on patients suffering from idiopathic dilated cardiomyopathy.[23-31] These studies suggest that H-FABP adds prognostic value over BNP in patients with decompensated HF.[26-28,30,31]

The only study of prognostic value of H-FABP in patients with stable CHF investigated 78 patients with idiopathic dilated cardiomyopathy who were stable for at least 3 months following an episode of decompensation.[402] It found that although higher H-FABP, BNP, TnT, myosin light chain-1 (MLC-1) and creatine kinase isoenzyme MB (CK-MB) levels were associated with a higher rate of combined cardiac death and HF hospitalisation, only BNP and MLC-1 were independent predictors of poor prognosis. However, patients with creatinine above 1.5mg/dL (133μmol/L) or Hb below 10g/dL were excluded from the study.

Our study is the first to include a large group of consecutive unselected stable CHF patients. The patients were also followed for a long period of time. We found that H-FABP level was not normally distributed and hence data analysis was more accurately performed after logarithmic transformation. A raised H-FABP was associated with higher long-term adverse event rates. After adjustment for factors that can affect H-FABP level, log[H-FABP] was an independent predictor of outcome in patients with stable HF in addition to the known prognostic markers including NT-proBNP, age, Hb, severity of LVSD, renal function, albumin and medication. Log[H-FABP] and log[NT-proBNP] had the same ability to predict 5-year outcomes.

The incremental prognostic value of log[H-FABP] in addition to log[NT-proBNP] was assessed using multiple methods as recommended by the American Heart Association.[365] Log[H-FABP] increased the performance of the logistic model as shown by an improvement in the calibration of the model. There was no change in the c-statistics as it is insensitive to the impact of adding a new predictor to a model.[367]Addition of log[H-FABP] also improved the IDI and NRI. We found only a modest correlation between log[H-FABP] and log[NT-proBNP], perhaps suggesting that elevated levels of H-FABP and BNP may be caused by different pathophysiological processes. Therefore, H-FABP may have incremental prognostic value in additional to BNP and help to stratify the risk of adverse event in the patients with stable HF.

5.1.4.1 Limitations

Although our study included only a small number of normal volunteers, their H-FABP levels were normally distributed and did not differ from a larger group of CVD patients whose H-FABP levels were also normally distributed.

We did not measure troponin levels since concomitant measurement of H-FABP and troponins has been investigated in previous studies.[22,402] Our study was not designed to establish any causal relationships between H-FABP level and other factors or explain the mechanism of H-FABP release in patients with stable HF due to LVSD. We also did not investigate the effect of treatment on H-FABP level or whether serial measurement of H-FABP may have a better prognostic value.

5.1.5 Conclusion

H-FABP level is raised in unselected patients with CHF due to LVSD who are on stable medication and the level is affected by renal function. After taking into consideration the factors that may affect its level, H-FABP has incremental prognostic value in addition to NT-proBNP.

5.2 Value of serial heart-type fatty acid-binding protein (H-FABP) measurements in patients with stable chronic heart failure due to left ventricular systolic dysfunction

5.2.1 Introduction

In patients hospitalised due to decompensated heart failure, persistently raised heart-type fatty acid-binding protein (H-FABP) despite treatment of heart failure (HF) and improvement in symptoms is associated with an increased risk of hospitalisation and cardiac death.[31,404] We have also found, in a group of ambulatory patients with HF, that a raised H-FABP after optimisation of treatment was associated with a worse survival. The change in H-FABP level in patients with chronic heart failure (CHF) on stable treatment has not been studied and whether serial measurement of H-FABP in stable CHF patients would help identify patients with higher risk of cardiovascular event is unknown.

Experience from studies using cardiac troponins in patients with stable CHF suggests that serial troponin measurement may help identify patients with a worse outcome. Sato et al was one of the first to show the potential value of serial troponin measurement in patients with CHF.[405] Three patterns of troponin measurement were identified in 60 patients with dilated cardiomyopathy: persistently normal level, normalised following a raised level and persistently raised level of troponin T (TnT). The patients with persistently raised TnT (n=17/60) had unfavourable myocardial remodelling, worsening of LV function and a higher cardiac event and mortality rate during the mean follow-up period of 15.9 ± 10.5 months.[405] Based on multiple serial TnT measurements in stable patients with CHF, Perna et al showed that the number of abnormal TnT measurements was an independent factor of a worse event-free survival after 18 months of follow-up.[406]

Using data from two large randomised controlled studies of patients with stable CHF (Valsartan Heart Failure Trial – ValHeFT and Gruppo Italiano per lo Studio della Sopravvivenza nell'Insufficienza Cardiaca-Heart Failure – GISSI-HF), Masson et al showed that changes in high-sensitivity TnT (hs-TnT) concentrations over time were robust predictors of cardiovascular events and may add prognostic discrimination in addition to conventional prognostic markers, including NT-proBNP.[390]

This section investigated the prevalence of persistently raised H-FABP and potential prognostic value of serial H-FABP measurement in patients with CHF.

5.2.2 Methods

5.2.2.1 Patients

Patients with stable CHF due to LVSD who took part in the previous cross-sectional study (n=483) were invited to return for a repeat blood test between 8 to 14 months following their initial blood test for H-FABP. In those who had an episode of decompensated heart failure or acute coronary syndrome, the samples were taken at least 4 weeks following the events.

5.2.2.2 Investigations

These patients were assessed and underwent investigations including blood tests and electrocardiogram as previously described. Additional blood samples were collected for analysis of H-FABP and NT-proBNP in the core laboratory as described early.

5.2.2.3 Follow-up

These patients had routine follow-up in the heart failure clinic annually, unless more frequent visit was deemed necessary.

5.2.2.4 Outcome measures

The primary outcome was the composite of heart failure hospitalisation and all-cause mortality. The secondary outcome was all-cause mortality.

5.2.2.5 Statistical analysis

Variables are presented and were analysed as described in Chapter 2.3. Paired binary variables were compared using McNemar's test and paired categorical variables were compared using Marginal Homogeneity test.

The dosage of loop diuretic was converted to furosemide equivalent dose (mg). The dosage of beta-blocker, ACEI, ARB and ARA was converted to percentage of the maximum recommended dose for HF treatment by the British National Formulary.

Any change in the H-FABP level during follow-up was determined. Raised H-FABP was defined as a level above 2.75ng/ml, which was 3 standard deviations above the mean H-FABP of a group of normal volunteers as previously described. Pearson's correlation was performed to identify any relationship between H-FABP and NT-proBNP at baseline and follow-up, and percentage change in H-FABP and NT-proBNP.

The patients were divided into 4 groups according to the normal level of 2.75 ng/ml: persistently low H-FABP (Group L), normalisation of H-FABP level (Group N), an increase in H-FABP during follow-up (Group I), persistently high H-FABP (Group H). Their outcome was compared. An arbitrary 25% change in H-FABP level was considered to be clinically relevant. The effect of a change in H-FABP level was investigated by dividing the patients into 3 groups according a decreased, unchanged or an increased H-FABP level followed by plotting a survival curve adjusted for baseline H-FABP level.

5.2.3 Results

Of the 483 patients, 231 patients returned for repeat blood tests and assessment within the study period. These 231 patients were included in this analysis.

Of the 252 patients who were not included in this analysis, 49 of them had died prior to or during the planned repeat blood sampling period and a further 25 patients did not have adequate blood sample data to be included in the analysis. The remaining 178 patients did not agree for repeat blood tests although they continued to have routine follow-up in the heart failure clinic. There were some differences in the baseline characteristics of the patients who were included in this analysis to those of the patients who were excluded. (Table 5.2.1) However, these differences did not affect the level of laboratory tests including H-FABP and NT-proBNP.

The 231 patients who were included in this analysis returned for a repeat blood test and assessment after a median period of 361 (323 – 377) days. H-FABP level reduced significantly over this period despite minimal change in the medications without any significant change in NYHA, severity of LVSD and other laboratory tests including NT-proBNP. (Table 5.2.2) There was significant but modest relationship found between the H-FABP and NT-proBNP levels at baseline and during repeat assessment. (Figure

5.2.1and Figure 5.2.2). The correlation between percentage change in H-FABP and NT-proBNP was also modest. (Figure 5.2.3)

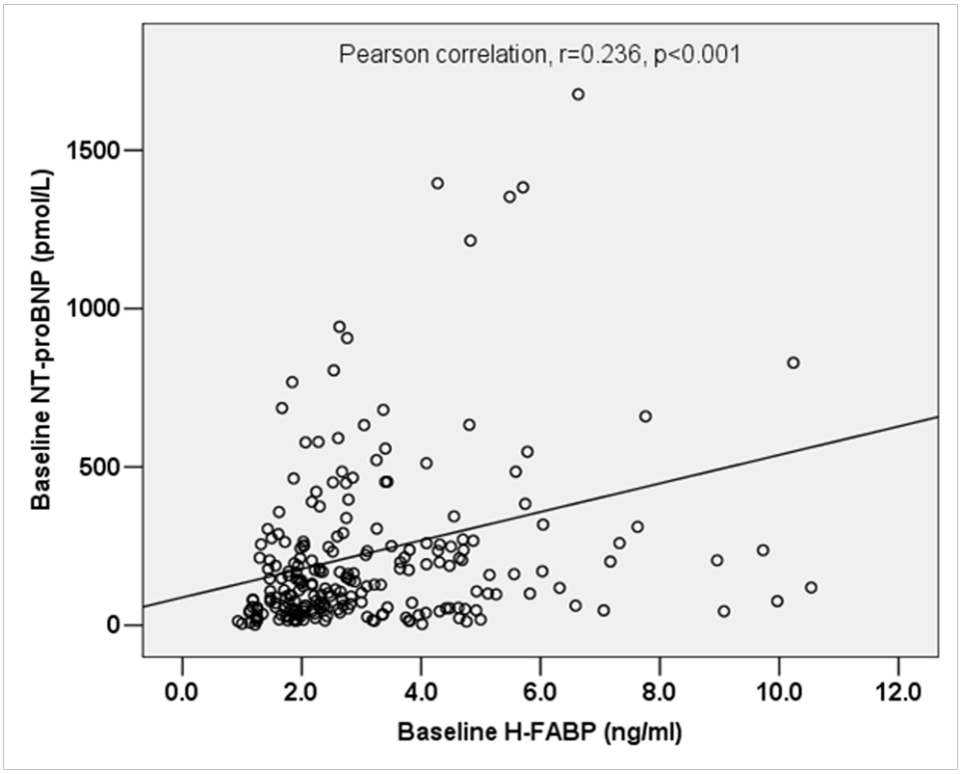


Figure 5.2.1 Correlation between baseline H-FABP and NT-proBNP

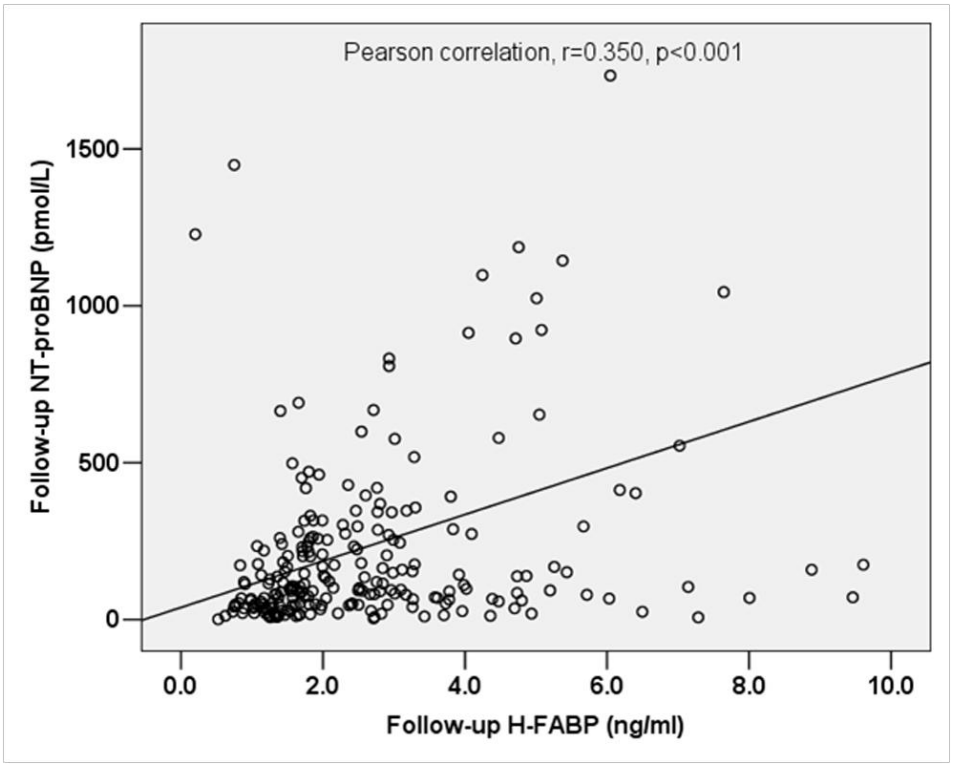


Figure 5.2.2 Correlation between follow-up H-FABP and NT-proBNP

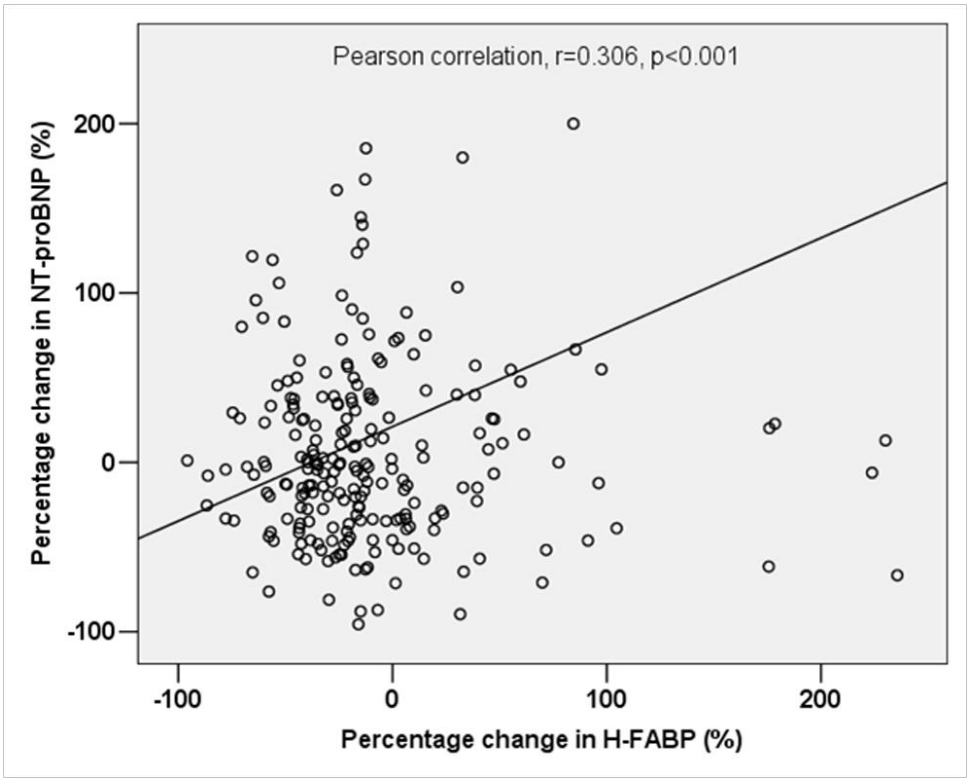


Figure 5.2.3 Correlation between the percentage change in H-FABP and that of NT-proBNP

Group H patients were older and taking lower dose of β-blocker than the other patients at the baseline. (Table 5.2.3) Compared to Group L and N, Group I and H patients required more diuretic at baseline and during repeat assessment. Group I and H patients also had higher H-FABP and NT-proBNP but lower GFR levels compared to those of Group L and H.

All the patients were followed for a median of 69.9 (65.4-73.3) months (range 62.7-81.4 months). During this period, 128 (55%) patients had had at least one hospitalisation due to cardiovascular cause and 71 (31%) patients were hospitalised due to decompensated heart failure; whilst 111 (48%) patients had died. Overall, 129 (55.8%) had died or had a hospitalisation due to decompensated heart failure.

By 5-years, 99 (43%) patients had died and 118 (51%) patients had died or had had at least a hospitalisation due to decompensated heart failure. Compared to the other patients, the patients who had a primary event by 5 years were older, had more severe LVSD and degree of breathlessness and required more loop diuretics but fewer of them were taking β-blocker. Patients who had a primary event also had higher H-FABP and NT-proBNP levels but lower Hb, GFR and albumin when compared to those who did not by 5 years. (Table 5.2.4)

Kaplan-Meier survival curves showed that patients with an increase in (Group I) or persistently high H-FABP (Group H) levels had a poorer long-term outcome than those who had persistently low (Group L) or normalisation of H-FABP (Group N) level. (Figure 5.2.4, and Table 5.2.5)

After adjustment for baseline H-FABP level, patients whose H-FABP level increased by 25% or more had the worst outcome when compared to those whose H-FABP level was unchanged or decrease by at least 25%. (Figure 5.2.5) The NT-proBNP levels for the patients whose H-FABP reduced by at least 25%, remained unchanged and increased by 25% or more were 203.8 ± 317.5, 233.4 ± 432.0 and 287.1 ± 364.9 pmol/L respectively at baseline (p=0.17) and 196.9 ± 301.7, 221 ± 357.8 and 365.6 ± 532.0 pmol/L respectively during repeat assessment (p=0.10).

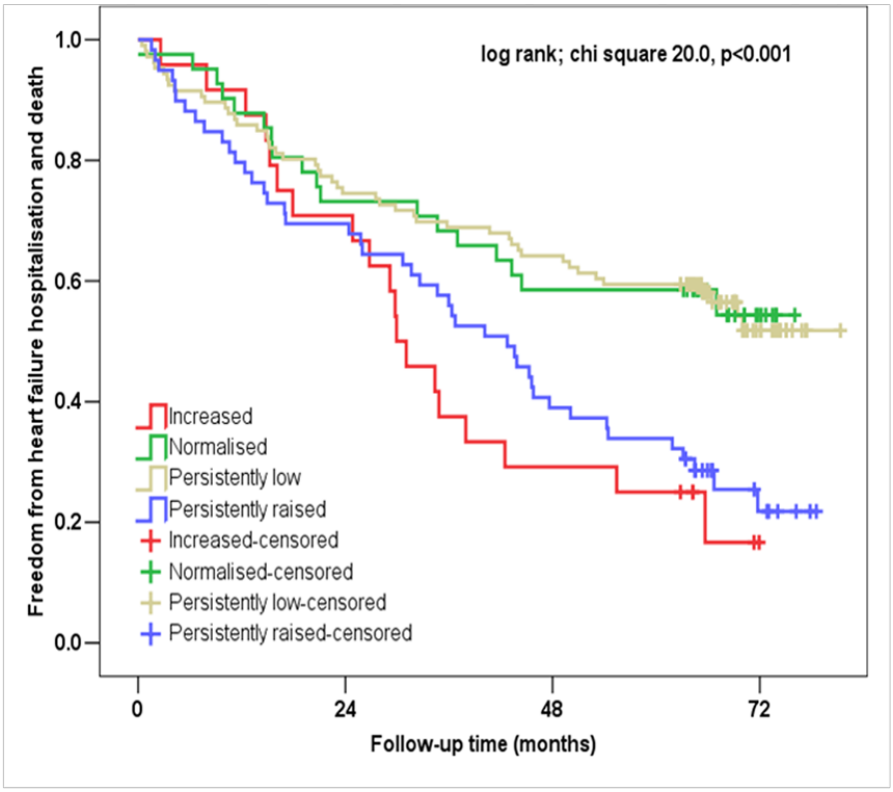


Figure 5.2.4 Kaplan Meier survival curves according to the change in H-FABP over follow-up period

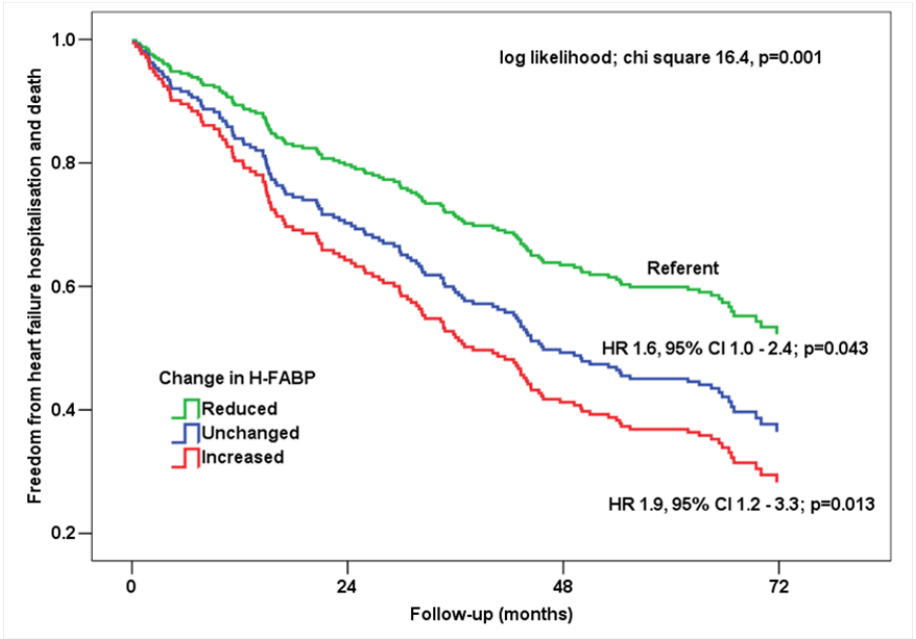


Figure 5.2.5 Survival curves in all patients according to the change in their H-FABP levels after adjusted for baseline H-FAPB

Table 5.2.1 Baseline characteristics of patients who were (n=231) and were not (n=252) included in the analysis

	Patients included n=231	Patients excluded n=252	All patients n=483
Age (years)	72 (64-77)	72 (65-77)	72 (64-77)
Man	83.5	73.4†	78.3
BMI (kg/m^2)	28.2 (25.5-31.3)	27.4 (24.6-31.5)	27.8 (25.0-31.5)
NYHA III/IV	22.9	19.8	21.3
Diabetes mellitus	26.8	19.5	23.0
Hypertension	39.4	42.1	40.8
Smoking history	76.6	74.2	75.4
IHD	82.3	73.0*	77.4
Other vascular disease	22.1	20.6	21.3
Atrial arrhythmia	25.5	35.7*	30.8
LVSD			
Mild	17.7	31.7†	25.1
Moderate	48.5	38.5	43.3
Severe	33.8	29.8	31.7
Medications:			
Loop diuretics	83.5	79.4	81.4
ACEIs or ARBs	90.9	91.7	91.3
β-blockers	87.9	81.3*	84.5
ARA	33.8	23.4*	28.4
Statins	60.2	51.2*	55.5
Digoxin	10.8	19.4†	15.3
Anti-platelets	52.4	49.2	50.7
Warfarin	29.0	33.3	31.3
Laboratory tests:			
<u>Baseline</u>			
H-FABP (ng/ml)	2.5 (1.9-3.9)	2.4 (1.8-3.4)	2.5 (1.8-3.7)
NT-proBNP (pmol/L)	125.9 (53.0-250.0)	130.0 (50.0-337.3)	127.8 (52.0-280.1)
GFR (ml/min/1.73m^2)	53.1 (38.0-68.5)	53.9 (40.2-67.6)	53.6 (39.5-68.2)

Haemoglobin (g/dL)	13.3 (12.2-14.1)	13.2 (12.0-14.2)	13.2 (12.1-14.1)
Sodium (mmol/L)	140 (138-141)	140 (138-141)	140 (138-141)
Albumin (g/L)	38(36-40)	38 (36-41)	38 (36-40)
Repeat			
H-FABP (ng/ml)	2.1 (1.6-3.9)	--	--
NT-proBNP (pmol/L)	182.0 (59.0-341.0)	--	--
GFR (ml/min/1.73m^2)	51.1 (38.6-66.6)	--	--
Haemoglobin (g/dL)	13.0 (11.7-14.2)	--	--
Sodium (mmol/L)	140 (139-142)	--	--
Albumin (g/L)	39 (36-41)	--	--

Continuous data are presented as median (inter-quartile range) and categorical data as percentage patient.

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; BMI, body mass index; GFR, glomerular filtration rate; H-FABP, heart type fatty acid binding protein; NT-proBNP, N-terminal proBNP; IHD, ischaemic heart disease.

When comparing patients who were included to those who were excluded from the analysis: * $p<0.05$ and † $p<0.01$; comparing baseline and repeat blood tests in patients included in this analysis: ‡ $p<0.05$, § $p<0.01$ and ‖ $p<0.001$

Table 5.2.2 The differences in NYHA, LVSD, medications and laboratory tests between the baseline and repeat assessment

	Baseline	Repeat assessment	*P*
BMI (kg/m^2)	28.2 (25.5-31.3)	27.8 (25.0-31.2)	0.18
NYHA III/IV	22.9	26.8	0.23
LVSD			0.17
Mild	17.7	23.4	
Moderate	48.5	44.2	
Severe	33.8	32.5	
Medications:			
Loop diuretics	83.5	85.7	0.27
ACEIs or ARBs	90.9	94.4	0.12
β-blockers	87.9	85.7	0.23
ARA	33.8	34.6	0.88
Statins	60.2	64.1	0.08
Digoxin	10.8	17.3	**0.001**
Anti-platelets	52.4	49.2	0.15
Warfarin	29.0	32.5	0.08
Dose equivalent:*			
Furosemide (mg)	63 ± 54	61 ± 48	0.69
ACEI (% maximum)	48 ± 37	48 ± 39	0.49
ARB (% maximum)	8 ± 25	13 ± 31	**0.002**
β-blockers (% maximum)	60 ± 45	61 ± 46	0.35
Laboratory tests:			
H-FABP (ng/ml)	2.5 (1.9-3.9)	2.0 (1.4-3.3)	**<0.001**
NT-proBNP (pmol/L)	125.9 (53.0-250.0)	104.0 (49.0-260.0)	0.73
GFR (ml/min/1.73m^2)	53.1 (38.0-68.5)	51.1 (38.6-66.6)	0.09
Haemoglobin (g/dL)	13.3 (12.2-14.1)	13.2 (12.0-14.2)	0.78
Sodium (mmol/L)	140 (138-141)	140 (138-141)	0.94
Albumin (g/L)	38(36-40)	38 (36-41)	0.67

Continuous data are presented as median (inter-quartile range) and categorical data as percentage patient.

*Dose equivalent are presented as milligram or percentage maximum recommended dose for treatment of heart failure

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; GFR, glomerular filtration rate; H-FABP, heart type fatty acid binding protein; NT-proBNP, N-terminal proBNP

Table 5.2.3 Characteristics of all patients divided into persistently low (Group L), normalisation of (Group N), an increase in (Group I) and persistently high (Group H) H-FABP levels during repeat assesssment

	Group L (n = 106)	Group N (n = 41)	Group I (n = 24)	Group H (n = 60)	*p*
Age (years)	68 ± 10	70 ± 11	70 ± 8	74 ± 9	**<0.001**
Men	81.1	85.4	83.3	86.7	0.81
BMI (kg/m^2)	29.0 ± 4.9	28.2 ± 5.2	30.1 ± 6.1	28.5 ± 5.0	0.58
NYHA III/IV	20.8	14.6	16.7	35.0	0.06
IHD	81.1	82.9	87.5	81.7	0.90
Hypertension	34.0	36.6	58.3	43.3	0.14
Diabetes	20.8	24.4	45.8	31.7	0.06
Atrial arrhythmias	18.9	29.3	29.2	33.3	0.18
Other vascular disease	19.8	17.1	41.7	21.7	0.10
LVSD					
Baseline:					0.10
Mild-to-moderate	22.6	12.2	4.2	18.3	
Moderate	45.3	48.8	41.7	56.7	
Severe	32.1	39.0	54.2	25.0	
Follow-up:					0.31
Mild-to-moderate	25.5	19.5	4.2	30.0	
Moderate	43.4	46.3	54.2	40.0	
Severe	31.1	34.1	41.7	30.0	
Medications					
Baseline:					
Loop diuretic	79.2	82.9	100	85.0	0.10
ACEI/ARB	93.4	90.2	95.8	85.0	0.26
ARA	30.2	36.5	33.3	38.3	0.73
β-blocker	86.8	97.6	91.7	81.7	0.10
Statin	57.5	70.7	75.0	51.7	0.10
Digoxin	7.5	12.2	4.2	18.3	0.12
Anti-platelet	55.7	41.5	62.5	50.0	0.32
Warfarin	25.5	29.3	29.2	35.0	0.64
Dose equivalent:					
Furosemide (mg)	56 ± 58	60 ± 45	82 ± 48	70 ± 53	**0.03**
ACEI (% maximum)	51 ± 37	47 ±37	34 ± 34	44 ± 40	0.63

ARB (% maximum)	9 ± 28	1 ± 8	14 ± 34	8 ± 24	0.25
β-blocker (% maximum)	61 ± 45	71 ± 40	63 ± 54	48 ± 43	**0.04**
Follow-up:					
Loop diuretic	79.2	82.9	100.0	93.3	**0.01**
ACEI/ARB	96.2	87.8	91.7	96.7	0.18
ARA	34.9	29.3	41.7	35.0	0.79
β-blocker	85.8	92.7	87.5	80.0	0.35
Statin	68.9	65.9	70.8	51.7	0.13
Digoxin	10.4	17.1	20.8	28.3	**0.03**
Anti-platelet	51.5	41.7	60.0	40.0	0.23
Warfarin	23.6	39.0	29.2	45.0	**0.03**
Dose equivalent:					
Furosemide (mg)	49 ± 45	60 ± 48	79 ± 50	75 ± 50	**0.001**
ACEI (% maximum)	53 ± 37	45 ± 40	36 ± 38	47 ± 40	0.19
ARB (% maximum)	13 ± 31	8 ± 24	22 ± 39	14 ± 31	0.29
β-blocker (% maximum)	65 ± 48	67 ± 42	58 ± 45	50 ± 44	0.10
Laboratory tests					
Baseline:					
H-FABP (ng/ml)	1.9 ± 0.44	4.2 ± 1.7	2.1 ± 0.5	5.1 ± 2.3	**<0.001**
NT-proBNP (pmol/L)	139 ± 149	205 ± 303	245 ± 239	405 ± 620	**<0.001**
GFR (ml/min/1.73m^2)	64 ± 17	54 ± 23	49 ± 21	40 ± 15	**<0.001**
Haemoglobin (g/dL)	13.5 ± 1.3	13.0 ± 1.5	13.0 ± 1.4	12.9 ± 1.7	0.05
Sodium (mmol/L)	140 ± 3	139 ± 2	139 ± 3	139 ± 3	0.74
Albumin (g/L)	39 ± 3	39 ± 3	38 ± 2	38 ± 3	0.11
Follow-up:					
H-FABP (ng/ml)	1.6 ± 0.5	1.9 ± 0.6	4.4 ± 1.7	4.5 ± 1.8	**<0.001**
NT-proBNP (pmol/L)	142 ± 179	192 ± 243	378 ± 536	377 ± 547	**0.002**
GFR (ml/min/1.73m^2)	62 ± 16	57 ± 19	43 ± 16	38 ± 13	**<0.001**
Haemoglobin (g/dL)	13.5 ± 1.4	13.2 ± 1.5	12.6 ± 1.6	12.7 ± 1.6	**0.001**
Sodium (mmol/L)	140 ± 3	139 ± 3	139 ± 3	139 ± 3	0.48
Albumin (g/L)	39 ± 3	38 ± 4	37 ± 4	37 ± 4	**0.003**

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; BMI, body mass index; GFR, glomerular filtration rate; H-FABP, Heart-type fatty acid-binding protein; LVSD, left ventricular systolic dysfunction; NYHA, New York Heart failure Association breathlessness classification; NT-proBNP, N-terminal proBNP

Table 5.2.4 Differences between patients who had a primary event (n=118) and those who had not (n=113) by 5 years

	Event-free n=118	With primary events n=113	*p*
Age (years)	67 (60-75)	74 (67-79)	**<0.001**
Man	88.5	78.8	**0.05**
BMI (kg/m^2)	28.5 (25.7-31.2)	28.0 (25.3-31.8)	0.72
NYHA III/IV	16.8	28.8	**0.03**
Diabetes mellitus	22.1	31.4	0.11
Hypertension	34.5	44.1	0.14
Smoking history	77.0	76.3	0.90
IHD	84.1	80.5	0.48
Other vascular disease	17.7	26.3	0.12
Atrial arrhythmia	22.1	28.8	0.24
LVSD:			
Baseline			**0.017**
Mild	21.2	14.4	
Moderate	54.0	43.2	
Severe	24.8	42.4	
Repeat			**0.001**
Mild	28.8	16.2	
Moderate	48.5	38.4	
Severe	22.7	45.5	
Medications:			
Baseline			
Loop diuretics	77.9	89.0	**0.02**
ACEIs or ARBs	92.0	89.8	0.56
β-blockers	92.0	83.9	0.58
ARA	27.4	39.8	**0.05**
Statins	67.3	53.4	**0.03**
Digoxin	12.4	9.3	0.49
Anti-platelets	56.6	48.3	0.21
Warfarin	24.8	33.1	0.17

Dose equivalent*:			
Furosemide (mg)	49 ± 40	77 ± 62	**<0.001**
ACEI (% maximum)	46 ± 37	49 ± 38	0.54
ARB (% maximum)	9 ± 26	7 ± 24	0.19
β-blockers (% maximum)	67 ± 43	53 ± 46	**0.006**
Follow-up			
Loop diuretics	78.8	92.4	**0.003**
ACEIs or ARBs	95.6	93.2	0.44
β-blockers	90.3	81.4	**0.03**
ARA	34.1	35.4	0.84
Statins	74.3	54.2	**0.001**
Digoxin	14.2	20.3	0.22
Anti-platelets	51.6	43.9	0.23
Warfarin	25.7	39.0	**0.03**
Dose equivalent*:			
Furosemide (mg)	49 ± 40	77 ± 62	<0.001
ACEI (% maximum)	46 ± 37	47 ± 39	0.72
ARB (% maximum)	15 ± 34	11 ± 28	0.53
β-blockers (% maximum)	69 ± 44	53 ± 47	**0.002**
Laboratory tests:			
Baseline			
H-FABP (ng/ml)	2.2 (1.8-3.4)	2.7 (2.1-4.5)	**<0.001**
NT-proBNP (pmol/L)	76.0 (36.5-169.5)	183.6 (83.0-377.4)	**<0.001**
GFR (ml/min/1.73m^2)	60.2 (48.1-74.1)	43.5 (31.8-57.2)	**<0.001**
Haemoglobin (g/dL)	13.6 (12.7-14.4)	12.8 (12.0-13.9)	**0.001**
Sodium (mmol/L)	139 (138-141)	140 (138-141)	0.92
Albumin (g/L)	39 (37-41)	38 (36-40)	**0.035**
Repeat			
H-FABP (ng/ml)	1.7 (1.3-2.7)	2.7 (1.7-4.0)	**<0.001**
NT-proBNP (pmol/L)	69.0 (34.0-141.8)	176.5 (90.3-394.8)	**<0.001**
GFR (ml/min/1.73m^2)	57.3 (44.2-75.4)	45.9 (34.5-55.4)	**<0.001**
Haemoglobin (g/dL)	13.6 (12.6-14.3)	12.8 (11.6-14.1)	**0.002**
Sodium (mmol/L)	140 (138-141)	139 (137-141)	0.43

Albumin (g/L)	39 (37-41)	38 (35-40)	**0.003**

Continuous data are presented as median (inter-quartile range) and categorical data as percentage patient.

*Dose equivalent are presented as milligram or percentage maximum recommended dose for treatment of heart failure

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; BMI, body mass index; GFR, glomerular filtration rate; H-FABP, heart type fatty acid binding protein; LVSD, left ventricular systolic dysfunction; NT-proBNP, N-terminal proBNP; NYHA, New York Heart Association breathlessness classification; IHD, ischaemic heart disease.

Table 5.2.5 Kaplan Meier freedom from death and heart failure hospitalisation by 5 years

H-FABP groups	*Freedom from primary event by 5 years (%)*
Persistently low (L) (n=106)	58.1 ± 4.8
Normalised (N) (n=41)	54.4 ± 8.2
Increased (I) (n=25)	25.5 ± 8.8
Persistently high (H) (n=59)	28.6 ± 5.9

5.2.4 Discussion

In patients with CHF who are on relatively stable treatment, H-FABP level decreases with time in the absence of significant change in NT-proBNP level or GFR. The relationship between H-FABP and NT-proBNP levels at any time point is only modest. Patients who experience a primary event have a higher H-FABP level compared to those who have not. The patients with an increase in or persistently raised H-FABP level have a poorer outcome than those who have a reduction in or persistently normal H-FABP levels. Regardless of the level of H-FABP at baseline or during repeat assessment, an increase in H-FABP level by more than 25% over a medium-term period confers a poorer prognosis.

This is the first study to investigate the dynamic change in H-FABP level in patients with chronic heart failure on stable medication. As discussed earlier, the only two previous studies investigated the dynamic change in H-FABP level involved patients with decompensated heart failure.[31,405] In these patients, the level of H-FABP decreases following stabilisation of the decompensated episode. However, our earlier analysis shows that in newly diagnosed ambulatory heart failure patients, the H-FABP level does not change despite a reduction in the NT-proBNP level following optimisation of their heart failure treatment. In contrast, this study demonstrates that in patients with CHF on stable medication, H-FABP level tends to decrease over a medium-term period in the absence of any significant change in the level of NT-proBNP, NYHA or the severity of LVSD.

Similar to the studies involving patients with decompensated heart failure[31,405], we found in patients with stable CHF that those with an increase in or persistently raised H-FABP level had a worse prognosis compared to those whose H-FABP level normalised or remained low during serial measurement. This is consistent to the studies of cardiac troponins.[390,407,408] Using data from 5284 patients in the Val-HeFT and GISSI-HF studies, Masson et al showed that the change in hs-TnT over 3 to 4 months is a robust predictors of cardiovascular events in patients with CHF.[390] The same study found that the change in hs-TnT level was strongly related to the change in NT-proBNP level. However, we found only modest correlation between the change in H-FABP level and the change in NT-proBNP level in our patients. This suggests that although low level of circulating cardiac troponins and H-FABP are both reflecting on-going myocardial damage in patients with CHF, their release may be mediated by different pathophysiological processes.

This study also found that irrespective of the baseline H-FABP level, an increase of subsequent H-FABP level by at least 25% confers a worse long-term prognosis in the patients with stable CHF; whilst those with a reduction of H-FABP level by 25% or more have a better prognosis. Importantly, the NT-proBNP levels at baseline and during repeat assessment were similar in these three groups of patients and in each of the group, the NT-proBNP levels did not change between the two measurements. Such finding is consistent with a prior study that a raised BNP level is associated with poorer outcome but once raised, any further changes in the BNP level does not alter the risk profile of patients with CHF.[408] Therapeutic strategy guided by B-type natriuretic peptide levels has improved treatment optimisation in patients with CHF when compared to conventional approach that is guided by patients' symptom. However, this strategy has not consistently translated into prognostic benefit.[8] Therefore serial measurement of H-FABP may be useful, at least as an

adjunctive to BNP or NT-proBNP measurement, in monitoring treatment response and identifying the patients who are at a higher risk of developing a cardiovascular event such that a more intensive therapeutic approach can be targeted at these patients.

As previously discussed, the mechanism(s) of H-FABP release in patients with stable CHF remains speculative and is thought to be related to on-going myocardial damage.[22,23] Among others, increased myocardial wall tension has been postulated as a mechanism of myocardial damage in heart failure. In isolated rat heart, an increase in preload can cause degradation of myofibril proteins independent of myocardial ischaemia.[409] Increased myocardial wall stretch due to ventricular overload can also induce cardiomyocyte apoptosis and necrosis.[410] This stretch-related cardiomyocyte damage can also occur through an integrin-mediated mechanism.[411] Clinically, raised serum BNP and troponin T and I has been shown to be associated with an elevated pulmonary capillary wedge pressure in patients with heart failure.[17,412] However, the modest correlation between H-FABP and NT-proBNP levels, and that H-FABP but not NT-proBNP decreases with time as observed in our study suggests that H-FABP release in patients with stable CHF may be predominantly related to other mechanisms that lead to on-going myocardial damage. In contrast, troponin and B-type natriuretic peptide release may be more closely related. Therefore, H-FABP may have prognostic value in addition to NT-proBNP in stratifying the risk of patients with stable CHF as observed in our previous study. For the same reason, H-FABP may be a better marker of on-going myocardial damage than cardiac troponins that could be used in combination with BNP to stratifying the risk of these patients.[22] In contrast to myofibril proteins, H-FABP is released rapidly from cardiomyocytes during myocardial injury even in the absence of myofibril damage or necrosis. As previously mentioned, H-FABP is detectable within 2 hours of acute myocardial infarction and peaks at about 4 to 6 hours before returning to baseline level within 20 hours.[188] Therefore, H-FABP may also be a more dynamic marker than cardiac troponins in detecting on-going myocardial damage.

5.2.4.1 Limitations and future

The number of patients in this study is small but they represent a typical cohort of unselected patients with CHF in actual clinical setting. As only patients who returned for a repeat blood test were included in the analysis, the study cohort might represent a group of patients with better prognosis. Nevertheless, the long-term survival observed in this study is in line with that observed in patients with CHF on modern heart failure treatment.[1,413] H-

FABP was only measured at two time points over a medium-term period and hence the trend of H-FABP in between these two measurements was unknown. The usefulness of multiple serial hs-TnT measurements in risk stratification for patients with CHF has been reported.[414] Therefore, larger cohort, more H-FABP measurements at shorter intervals and concomitant hs-TnT measurement should be the scope of future studies.

5.2.5 Conclusion

In patients with chronic heart failure on stable treatment, H-FABP levels decrease with time without any significant change in the level of NT-proBNP. Patients with an increase in or persistently raised H-FABP level have a worse long-term prognosis.

5.3 The effect of heart failure treatment optimisation on heart-type fatty acid-binding protein (H-FABP) level in patients with heart failure due to left ventricular systolic dysfunction.

5.3.1 Introduction

In patients hospitalised due to decompensated heart failure, treatment of heart failure (HF) improves symptoms and reduces the level of heart-type fatty acid-binding protein (H-FABP).[31,404] However, some patients continue to have an increased H-FABP level despite an improvement in the signs and symptoms of HF.[31] These patients with persistently high H-FABP have an increased risk of hospitalisation for HF and cardiac death when compared to those who have a reduction in the H-FABP level.[31]

The pathophysiology for persistently increased H-FABP is unclear but these patients are generally older, have more severe HF symptoms and a worse left ventricular function, and require more diuretics suggestive that they suffer from a more advanced disease. The persistently high H-FABP may indicate that subclinical myocardial damage continues during the compensated stage of heart failure despite a satisfactory clinical response to treatment. However, whether the same can be observed in ambulatory HF patients who undergo initiation and/or optimisation of their HF therapy is unknown.

Therefore, in a group of ambulatory patients with newly diagnosed HF due to left ventricular systolic dysfunction (LVSD) undergoing initiation or optimisation of their treatment, we investigated the effect of treatment on the level of H-FABP and whether a change in the level of H-FABP can be related to their prognosis.

5.3.2 Methods

5.3.2.1 Patients

Patients who were found to have HF due to LVSD following assessment in the heart failure service in Hull and East Yorkshire Hospitals NHS Trust and those referred for initiation or optimisation of their treatment following the diagnosis of HF due to LVSD were recruited. Those who had an episode of decompensated heart failure or acute coronary syndrome within the previous 4 weeks were excluded. All patients gave informed written consent.

5.3.2.2 Investigations

These patients were assessed and underwent investigations including blood tests, electrocardiogram and echocardiogram at baseline and during follow-up as previously described in Chapter 2.2.2 and 2.2.3. Specifically, blood samples were collected for analysis of H-FABP and NT-proBNP in the core laboratory as described in Chapter 2.2.3.

5.3.2.3 Follow-up

The treatment strategy was to achieve optimal heart failure therapy for all the patients within the first four months of their initial presentation to the clinic. The patients were reassessed three months after achieving stable and optimal heart failure therapy as described above. These patients were then followed up four monthly for the first year from their initial presentation and then annually, unless more frequent visit was deemed necessary.

5.3.2.4 Outcome measures

The primary outcome was the composite of heart failure hospitalisation and all-cause mortality. The secondary outcomes were HF hospitalisation and all-cause mortality.

5.3.2.5 Statistical analysis

Variables are presented and were analysed as described in Chapter 2.3 Any change in the H-FABP level during follow-up was determined. Raised H-FABP was defined as a level above 2.75ng/ml, which was 3 standard deviations above the mean H-FABP of a group of normal volunteers as previously described. The proportions of patient with a raised H-FABP at presentation and during follow-up were compared. The dosage of loop diuretic was converted to furosemide equivalent dose (mg). The dosage of beta-blocker, ACEI, ARB and ARA was converted to percentage of the maximum recommended dose for HF treatment by the British National Formulary. Subgroup analyses were performed in the patients who were not taking a beta-blocker or ACEI at baseline in order to investigate the effect of these medications had on the level of H-FABP. Spearman's rho coefficient was used to identify the correlations between H-FABP and NT-proBNP level.

To identify any relationship of a change in H-FABP level had on the outcome and left ventricular (LV) systolic function, the patients were divided into 4 groups according to the normal level of 2.75 ng/ml: persistently low H-FABP (Group L), normalisation of H-FABP level (Group N), an increase in H-FABP during follow-up (Group I), persistently high H-FABP (Group H). Their outcome and change in LV systolic function were compared.

5.3.3 Results

Sixty patients were recruited. Their median age was 71 (67 – 76) years and the majority were men and had IHD. (Table 5.3.1) Only approximately half of the patients were on a β-blocker or an ACEI or ARB. Following optimisation of heart failure treatment, the patients were reassessed 4.1 (3.9 – 5.6) months later (range 3.2 – 8.9 months). Eighty or more percent of the patients were taking a β-blocker or an ACEI or ARB during reassessment with a corresponding increase in the dosage of these medications. (Table 5.3.2)

Table 5.3.1 Baseline characteristics of all patients divided into previously low (Group L), normalisation of (Group N), an increase in (Group I) and persistently high (Group H) H-FABP lelevs during follow-up according to the cut-off threshold of 2.75 ng/ml

	Group L (n = 28)	Group N (n = 11)	Group I (n = 9)	Group H (n = 12)	All (n = 60)
Age (years)	70 ± 8	69 ± 8	74 ± 7	74 ± 8	71 ± 8
Men	61	91	78	50	67
BMI (kg/m^2)	30 ± 6	27 ± 5	27 ± 5	28 ± 6	27 (24-31)
NYHA III/IV	29	9	33	33	27
IHD	86	73	89	67	80
Hypertension	50	46	67	75	57
Diabetes	22	9	11	33	20
AF	25	55	0	26	27
NCVD	18	36	22	25	23
LVSD					
Mild-to-moderate	21	36	22	25	25
Moderate	43	27	56	50	43
Severe	36	36	22	25	32
LVEF (%)	31 ± 6	32 ± 9	35 ± 12	31 ± 6	32 ± 8
Loop diuretic	82	64	100	92	83
ACEI	46	64	67	25	48
ARB	4	9	0	8	5
ACEI/ARB	50	73	67	33	53
ARA	18	9	11	33	18.3
β-blocker	50	46	44	83	55
Statin	46	55	56	58	52
Anti-platelet	61	27	89	58	58
Sodium (mmol/L)	141 ± 3	139 ± 8	140 ± 2	141 ± 2	140 ± 3
Urea (mmol/L)†	**6.5 ± 2.3**	**9.8 ± 5.5**	**8.0 ± 2.3**	**11.1 ± 5.3**	**8.2 ± 4.1**
Creatinine (μmol/L)†	**101 ± 22**	**164 ± 119**	**110 ± 26**	**162 ± 63**	**126 ± 66**
GFR ($ml/min/1.73m^2$)*	**66 ± 20**	**56 ± 9.2**	**57 ± 11**	**36 ± 13**	**57 ± 19**
Albumin (g/L)	39 ± 3	38 ± 5	37 ± 4	37 ± 4	38 ± 4

Haemoglobin (g/dL)	13.6 ± 1.5	13.3 ± 2.0	13.9 ± 1.4	12.6 ± 2.0	13.4 ± 1.7
RDW (%)	14.6 ± 1.4	14.9 ±2.3.4	14.9 ± 1.6	15.4 ± 2.2	14.9 ± 1.8
H-FABP (ng/ml)*	**2.0 ± 0.45**	**4.6 ± 3.2**	**2.1 ± 0.3**	**4.1 ± 1.8**	**2.9 ± 2.0**
NT-proBNP (pmol/L)	271 ± 265	387 ± 485	345 ± 192	887 ± 976	426 ± 557
hs-CRP (mg/L)	5.3 ± 4.6	6.9 ± 8.2	9.1 ± 9.8	10.7 ± 10.8	7.2 ± 7.8

Continuous data are presented in median (inter-quartile range) and categorical data in percentage of patients.

* $p<0.001$ and † $p<0.01$

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; AF, atrial arrhythmia; BMI, body mass index; GFR, glomerular filtration rate; IHD, ischaemic heart disease; H-FABP, heart-type fatty acid-binding protein; hs-CRP, high-sensitivity c-reactive protein; LVEF, left ventricular ejection fraction; LVSD, left ventricular systolic dysfunction; NCVD, non-cardiac vascular disease; NT-proBNP, N-terminal pro-brain natriuretic peptide; RDW, red cell distribution width.

Table 5.3.2 The changes in medications, symptom, LV function and laboratory tests following optimisation of heart failure treatment

	Baseline (n = 60)	Reassessment (n = 60)	*P*
NYHA III/IV	27	20	0.50
LVSD			0.91
Mild-to-moderate	25	29	
Moderate	43	43	
Severe	32	28	
LVEF (%)	32 ± 8	32 ± 8	0.51
Loop diuretic	83	83	1.00
Furosemide equivalent dose (mg)	57 ± 25	56 ± 45	0.19
ACEI	**48**	**73**	**0.001**
Percentage maximum ACEI dose (%)	**28 ± 38**	**48 ± 41**	**0.002**
ARB	**5**	**15**	**0.03**
Percentage maximum ARB dose (%)	**2 ± 13**	**11 ± 30**	**0.01**
ACEI/ARB	**53**	**88**	**<0.001**
ARA	18	27	0.23
β-blocker	**55**	**80**	**<0.001**
Percentage maximum β-blocker dose (%)	**26 ± 37**	**46 ± 38**	**<0.001**
Statin	52	55	0.69
Anti-platelet	58	55	0.75
Sodium (mmol/L)	140 ± 3	140 ± 3	0.22
Urea (mmol/L)	**8.2 ± 4.1**	**9.4 ± 5.1**	**0.048**
Creatinine (μmol/L)	126 ± 66	128 ± 52	0.08
GFR (ml/min/1.73m^2)	**57 ± 19**	**54 ± 21**	**0.049**
Albumin (g/L)	38 ± 4	37 ± 4	0.49
Haemoglobin (g/dL)	**13.4 ± 1.7**	**13.0 ± 1.6**	**0.03**
RDW (%)	14.9 ± 1.8	14.3 ± 1.3	0.05
H-FABP (ng/ml)	2.9 ± 2.0	2.8 ± 1.5	0.91
H-FABP ≥ 2.75 ng/ml	40	35	0.82
NT-proBNP (pmol/L)	**226 ± 557**	**337 ± 631**	**0.004**
hs-CRP (mg/L)	7.2 ± 7.8	13.5 ± 25.0	0.74

Continuous data are presented in mean ± standard deviation and categorical data in percentage of patients.

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; AF, atrial arrhythmia; BMI, body mass index; GFR, glomerular filtration rate; IHD, ischaemic heart disease; H-FABP, heart-type fatty acid-binding protein; hs-CRP, high-sensitivity c-reactive protein; LVEF, left ventricular ejection fraction; LVSD, left ventricular systolic dysfunction; NCVD, non-cardiac vascular disease; NT-proBNP, N-terminal pro-brain natriuretic peptide; RDW, red cell distribution width.

Considering all the patients as a group, the level of H-FABP did not change. (Table 5.3.2) Overall, 23 (38%) patients and 21 (35%) patients had a raised H-FABP ≥ 2.75 ng/ml at baseline and during follow-up respectively, p = 0.82. However, 11 (18%) patients had normalisation of H-FABP level (Group N, 4.60 ng/ml vs 2.03 ng/ml, p=0.022) whilst 9 (15%) patients developed an increased H-FABP level (Group I, 2.14 ng/ml vs 4.45 ng/ml, p=0.002) during follow-up; whilst 28 (47%) patients had persistently low H-FABP (Group L, 1.96 ng/ml vs 1.89 ng/ml, p=0.57) and 12 (20%) patients had persistently high H-FABP (Group H, 4.11 ng/ml vs 4.42 ng/ml, p=0.47). (Figure 5.3.1) In contrast, the NT-proBNP significantly reduced during follow-up when all the patients were considered as a group (Table 5.3.2) but did not change significantly when the patients were divided into the 4 groups according to the change in their H-FABP levels. (Figure 5.3.2)

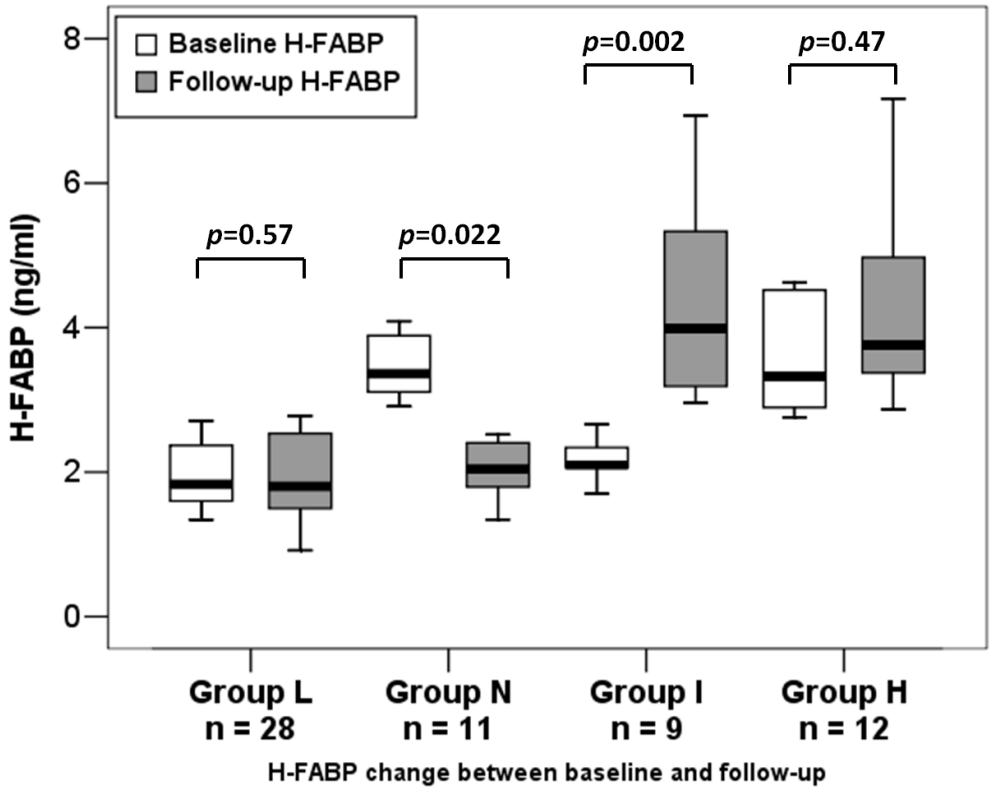


Figure 5.3.1 The change in H-FABP levels following optimisation of heart failure treatment (n=60)

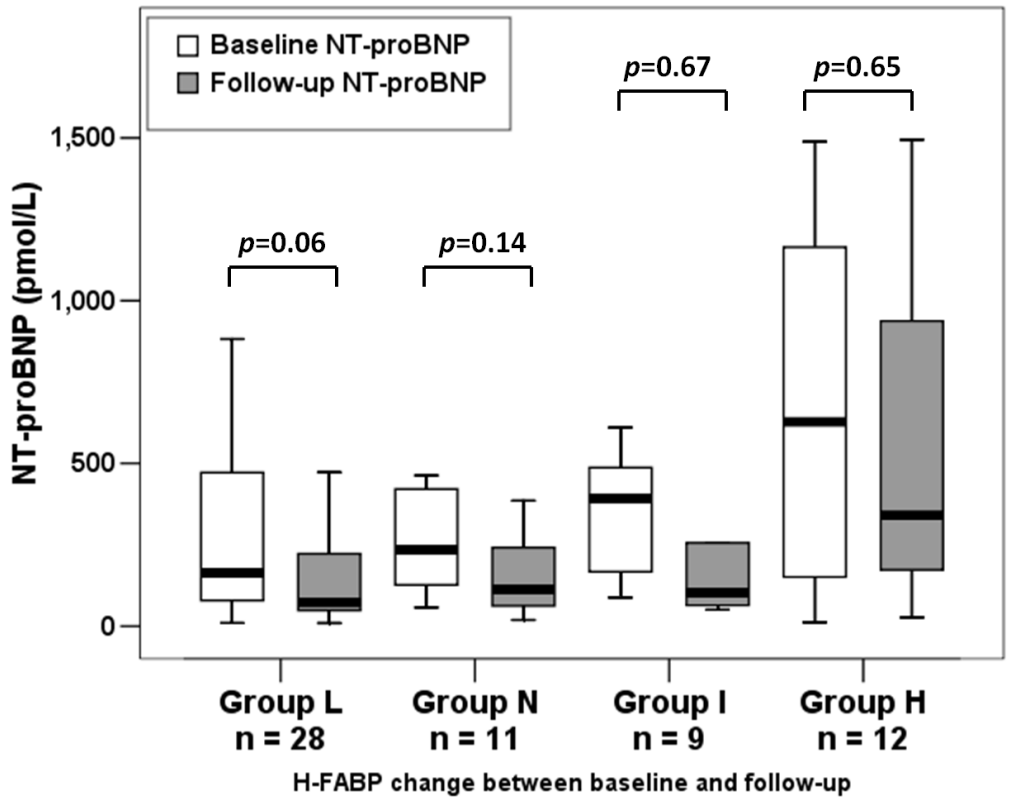


Figure 5.3.2 The change in NT-proBNP levels following optimisation of heart failure treatment (n=60)

Considering all the patients as a group, there was only modest correlation between the baseline H-FABP and NT-proBNP levels ($r = 0.33$, $p<0.001$); and the follow-up H-FABP and NT-proBNP level ($r = 0.30$, $p<0.001$). The change in H-FABP level from baseline to follow-up was not related to that of NT-proBNP ($r = -0.02$, $p=0.91$).

During follow-up, patients who had persistently high H-FABP or an increase in H-FABP (Group H or I) had a worse renal function and higher NT-proBNP compared to those who had persistently low or normalisation of H-FABP level (Group L or N). (Table 5.3.3) Group H and I also required higher dose of loop diuretic but could tolerate lower dose of ACEI with more of them taking an ARA than Group L and N.

The left ventricular systolic function did not change during the follow-up period. (Table 5.3.3) The change in H-FABP levels during follow-up was not associated with any change in the left ventricular function. (Figure 5.3.3)

Table 5.3.3 NYHA, LV function, medications and laboratory variables of all patients divided into persistentl low (Group L), normalisation of (Group N), an increase in (Group I) and persistently high (Group H) H-FABP levels during follow-up accod=rding to the cut-off threshold of 2.75 ng/ml

	Group L (n = 28)	Group N (n = 11)	Group I (n = 9)	Group H (n = 12)	*P*
NYHA III/IV	21	18	11	25	0.88
LVSD					0.80
Mild-to-moderate	36	28	22	17	
Moderate	36	36	56	58	
Severe	28	36	22	25	
LVEF (%)	31 ± 8	34 ± 12	32 ± 8	31 ± 5	0.96
Loop diuretic	**82**	**55**	**100**	**100**	**0.01**
Furosemide equivalent dose (mg) (%)	**46 ± 43**	**36 ± 38**	**80 ± 45**	**78 ± 44**	**0.02**
ACEI	79	73	89	50	0.18
Percentage maximum ACEI dose (%)	**50 ± 39**	**61 ± 44**	**68 ± 40**	**15 ± 28**	**0.01**
ARB	14	18	0	25	0.45
Percentage maximum ARB dose (%)	14 ± 36	11 ± 30	0	13 ± 29	0.51
ACEI/ARB	93	91	89	75	0.44
ARA	**14**	**36**	**11**	**58**	**0.02**
β-blocker	75	73	89	92	0.52
Percentage maximum β-blocker dose (%)	46 ±38	45 ± 46	43 ± 31	46 ± 39	0.99
Statin	54	46	79	50	0.49
Anti-platelet	61	36	67	50	0.47
Sodium (mmol/L)	140 ± 2	140 ± 3	138 ± 3	139 ± 3	0.69
Urea (mmol/L)	**7.3 ± 2.9**	**6.8 ± 2.3**	**12.6 ± 8.1**	**14.2 ± 3.9**	**<0.001**
Creatinine (μmol/L)	**108 ± 32**	**114 ± 18**	**127 ± 44**	**189 ± 70**	**<0.001**
GFR (ml/min/1.73m^2)	**62 ± 23**	**59 ± 11**	**53 ± 16**	**32 ± 13**	**<0.001**
Albumin (g/L)	38 ± 3	40 ± 3	35 ± 3	37 ± 4	0.02

Haemoglobin (g/dL)	12.9 ± 1.5	13.7 ± 1.4	13.2 ± 1.8	12.2 ± 1.8	0.12
RDW (%)	14.3 ± 1.1	14.3 ± 1.4	13.8 ± 1.1	14.8 ± 1.7	0.57
H-FABP (ng/ml)	**1.9 ± 0.5**	**2.0 ± 0.4**	**4.5 ± 1.5**	**4.4 ± 1.6**	**<0.001**
NT-proBNP (pmol/L)	**174 ± 218**	**158 ± 115**	**470 ± 783**	**781 ± 1109**	**0.02**
hs-CRP (mg/L)	20.7 ± 36.1	5.4 ± 4.3	9.6 ± 16.5	9.4 ± 9.3	0.38

Continuous data are presented in mean ± standard deviation and categorical data in percentage of patients.

ACEI, angiotensin converting enzyme inhibitor; ARA, aldosterone receptor antagonist; ARB, angiotensin receptor blocker; AF, atrial arrhythmia; BMI, body mass index; GFR, glomerular filtration rate; IHD, ischaemic heart disease; H-FABP, heart-type fatty acid-binding protein; hs-CRP, high-sensitivity c-reactive protein; LVEF, left ventricular ejection fraction; LVSD, left ventricular systolic dysfunction; NCVD, non-cardiac vascular disease; NT-proBNP, N-terminal pro-brain natriuretic peptide; RDW, red cell distribution width.

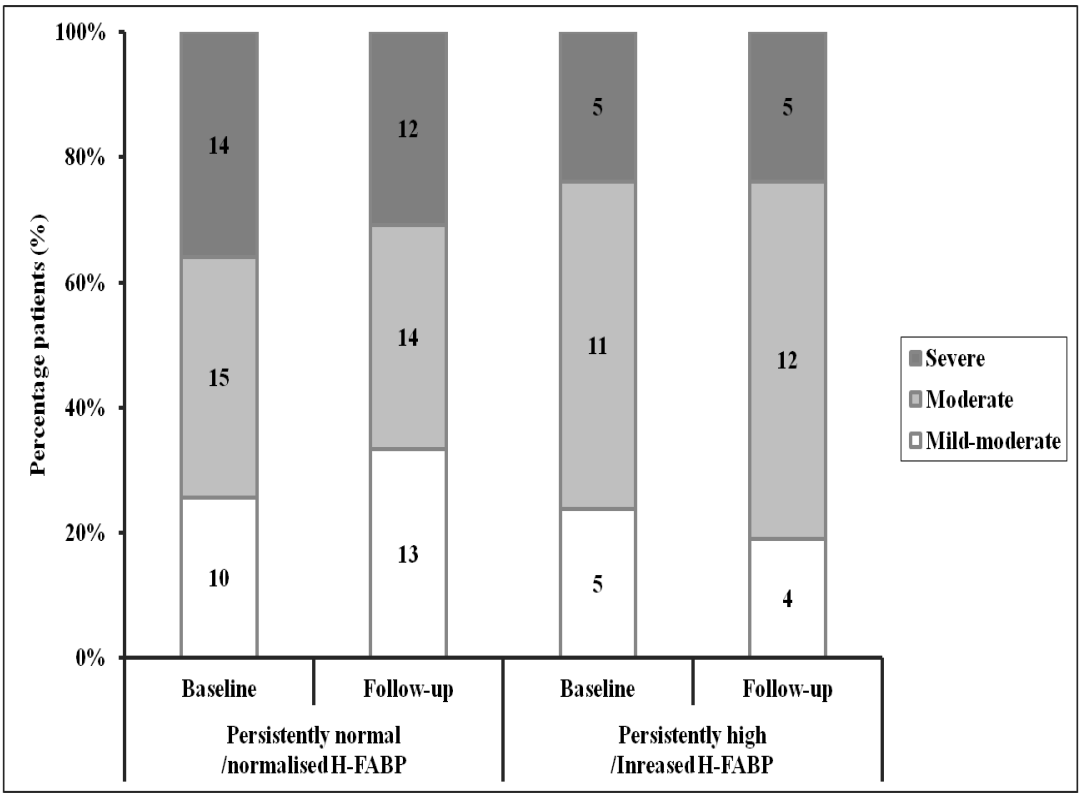


Figure 5.3.3 The degree of LV dysfunction at the baseline and during follow-up in the patients with persistently normal or normalised H-FABP level (n=39) and those with persistently high or an increase in H-FABP level (n=21)

All the patients were then follow-up for a mean period of 76.2 ± 5.3 (range 67.5 – 76.2) months during which 33 (55%) patients had died, 17 (28%) patients had at least a hospitalisation for decompensated heart failure and 37 (62%) had either died or at least a heart failure hospitalisation. The survivors were followed for 51.4 ± 27.8 (range 0.3 – 84.7) months. Group H and I had higher long-term mortality or combined mortality and HF hospitalisation when compared to Group L and N. (Figure 5.3.4, Figure 5.3.5 and Figure 5.3.6)

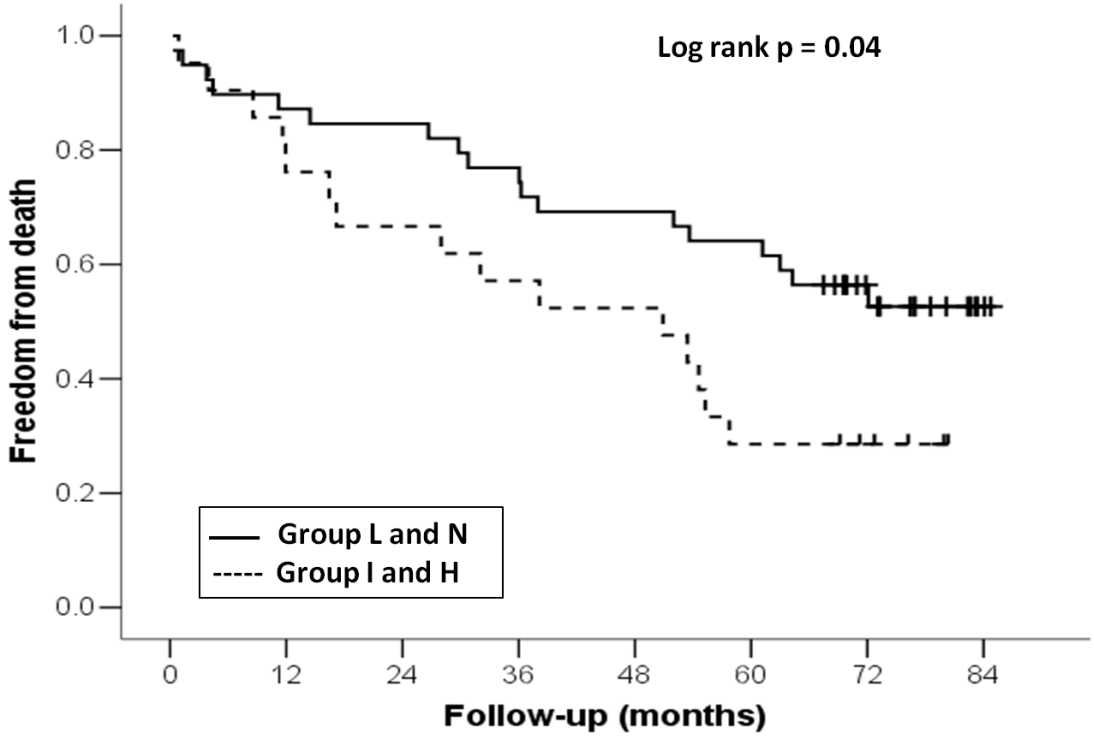


Figure 5.3.4 Kaplan Meier Curves for long-term mortality for patients with persistently normal or normalisation of H-FABP level (Group L and N) and patients who had an increase in or persistently high H-FABP level (Group I and H) following optinmisation of heart failure treatment

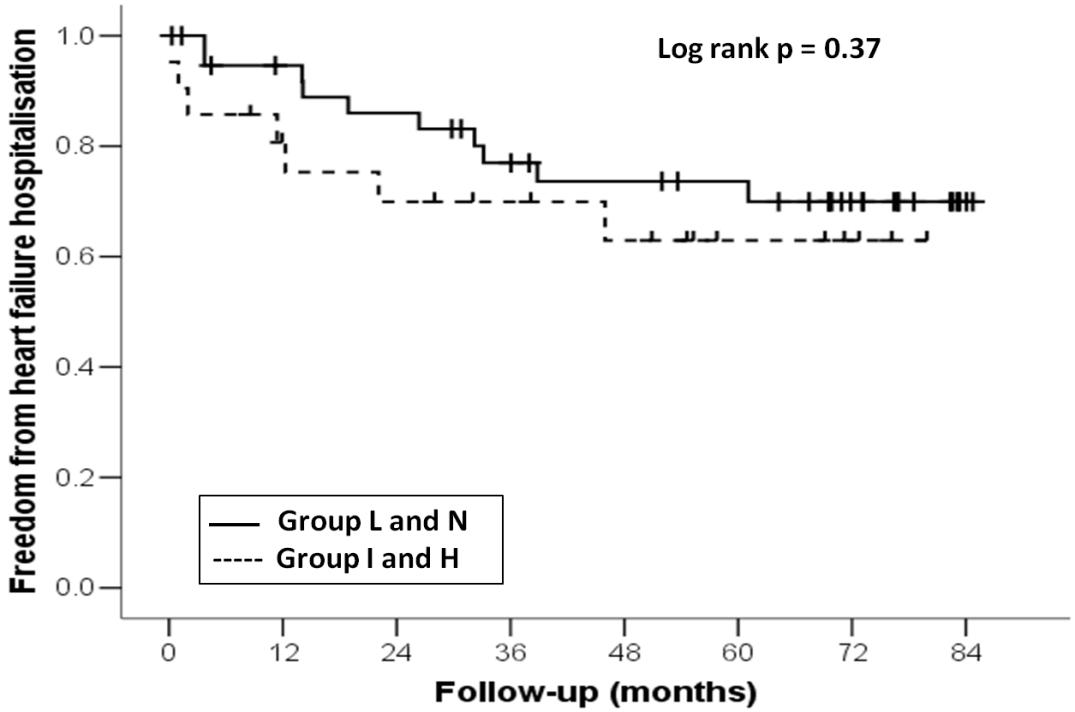


Figure 5.3.5 Kaplan Meier curves for long-term heart failure hospitalisations for the patients with persistently normal or normalisation of H-FABP level (Group L and N) and patients who had an increase in or persistently high H-FABP level (Group I and H) following optimisation of heart failure treatment

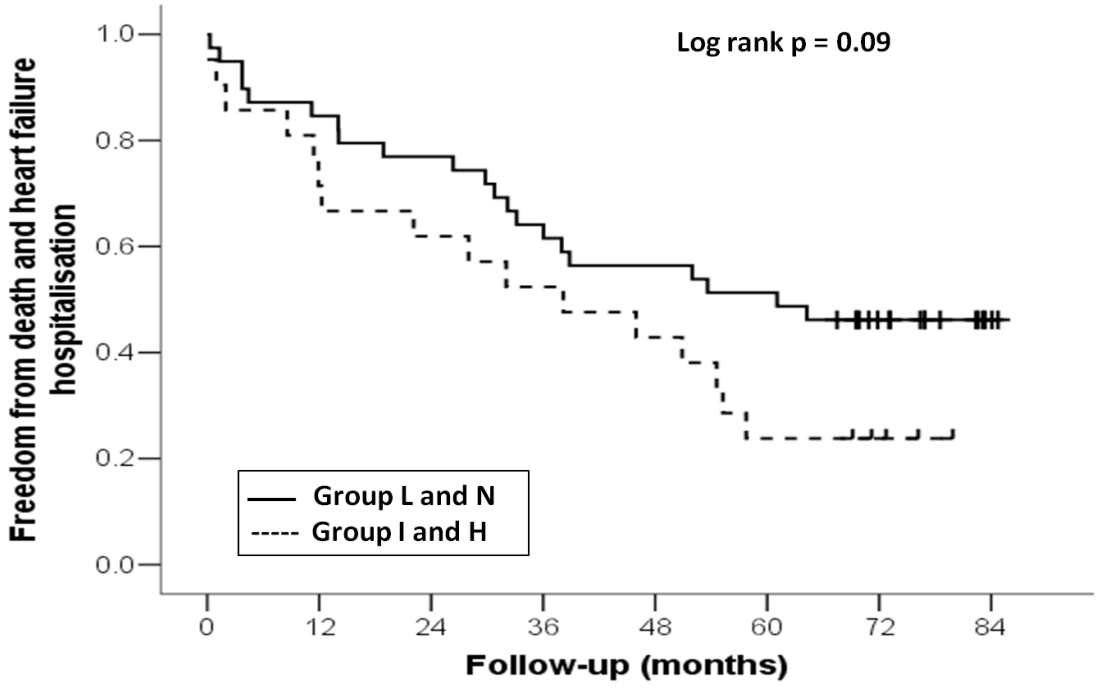


Figure 5.3.6 Kaplan Meier curves for long-term combined death and heart failure hospitalisations for the patients with persistently normal or normalisation of H-FABP level (Group L and N) and patients who had an increase in or persistently high H-FABP level (Group I and H) following optimisation of heart failure treatment

5.3.4 Discussion

This study shows that following initiation and optimisation of heart failure treatment in ambulatory CHF patients, H-FABP does not change but NT-proBNP decreases. However, a subgroup of patients have an increase in or persistently high H-FABP level and these patients have a higher mortality compared to those who have normalisation or persistently normal H-FABP level even without any significant change in their NT-proBNP level.

This study is the first to investigate the change in H-FABP level and its association with prognosis in ambulatory heart failure patients. The only two other studies of serial H-FABP measurement involved patients hospitalised for acute decompensated heart failure.[31,404] In patients with decompensated heart failure, Niizeki et al found that 51 of their 113 patients had persistently high H-FABP level despite stabilisation of their decompensated episode.[31] These patients had a much higher risk of cardiac death and rehospitalisation than those who had normalisation of or persistently normal H-FABP level. In our ambulatory heart failure patients, we found that in addition to a group of patients with persistently high H-FABP, some patients' H-FABP level increased despite optimisation of their heart failure therapy. Together, these patients had a higher mortality compared to those who had normalisation of or persistently normal H-FABP level.

Goto et al found that in 48 consecutive patients hospitalised for decompensated heart failure, the level of H-FABP and BNP decreased following the treatment of decompensated heart failure.[404] In both Niizeki's and Goto's studies, the reduction in H-FABP following stabilisation of heart failure correlated with the decrease in BNP level[31,404] In contrast, we did not find any relationship between the change in H-FABP level and that of NT-proBNP following optimisation of heart failure therapy. However, we found a modest correlation between H-FABP and NT-proBNP at baseline and during follow-up which is consistent with previous studies.[31,404] Patients with persistently high H-FABP (Group H) also had a higher level of NT-proBNP level. Therefore, it is unclear whether the increase in H-FABP and NT-proBNP are directly related or they involve in distinct pathophysiological processes.

A raised H-FABP level in patients with CHF may be related to on-going myocardial damage.[23] This can occur even in clinical stable CHF patients.[402] In fact, virtually all CHF patients have detectable level of cardiac troponins.[11,415] As much as 86% of the patients enrolled in the Valsartan Heart failure Trial (Val-HeFT) and 98% of those in

Gruppo Italiano per lo Studio della Sopravvivenza nell'Insufficienza Cardiac-Heart Failure (GISSI-HF) had detectable high-sensitivity troponin levels.[390] Post-mortem study of the heart of CHF patients who had raised circulating troponin has shown patchy fibrosis and degenerative myocyte changes.[12] Degeneration of hypertrophied myocytes has also been observed in human heart.[416]

The mechanism(s) of H-FABP or troponin release or clearance in patients with CHF remains speculative.[417,418] Microcirculatory abnormalities[419] and reduction in subendocardial perfusion[420] have been implicated. Others including increased myocardial wall stress, neurohormonal activation, oxidative stress, inflammation and altered calcium handling have also been advocated.[421] These factors can promote myocyte damage or death by producing either myocyte necrosis[422] or apoptosis[423]. Indeed, activated TNF and Fas/Fas ligand system that play a role in apoptosis has been found to be associated with a raised H-FABP in patients with CHF.[14] However, renal dysfunction is common in CHF[401] and a reduction in the clearance of H-FABP or troponins may also play a part.[424] This is consistent with our findings that patients with an increased or persistently high H-FABP (Group I and H) had a worse renal function than the others.

In patients with acute decompensated heart failure, treatment of heart failure was associated with an improvement in left ventricular systolic function and reduction in H-FABP.[31,404] However, we did not find a relation between the change in H-FABP level and LV systolic function. This may be related to small patient cohort and the relatively short interval between the baseline and follow-up echocardiographic assessment.

Consistent with the study that involved patients with decompensated heart failure[31], the mortality was higher in those patients who developed or had persistently high H-FABP (Group I and H) . Whether this was related to on-going myocardial damage is unclear. It may be just that these were patients with a more advanced disease or suffering from multiple co-morbidities since they had a worse renal function, required higher dose of diuretic and could tolerate lower dose of ACEI.

The change in H-FABP was associated with long-term outcome in the absence of significant change in NT-proBNP suggests that H-FABP may help to monitor treatment response and stratify the risk of patients so that patient with a poorer predicted outcome can be targeted for more intensive treatment regimen.

5.3.4.1 Limitations

The patient cohort in this study is small. Therefore, whether the change in H-FABP has incremental prognostic value over NT-proBNP could not be established. The effects of individual type of heart failure medication such β-blocker or ACEI and ARB was not investigated. Cardiac troponin was not performed in the study as previously described.

5.3.5 Conclusion

Despite optimisation of heart failure treatment, a proportion of CHF patients developed or had persistently high H-FABP level. These patients had a worse long-term prognosis.

Chapter 6 Enhanced External Counterpulsation in Chronic Heart Failure

6.1 Impact of enhanced external counterpulsation on symptoms, health status and medication use in patients with both angina and heart failure.

The results from this chapter have been presented in the European Society of Cardiology – Heart Failure update.[425]

6.1.1 Introduction

Most patients with heart failure have ischaemic heart disease, many have angina and some have severe angina unresponsive to medical therapy. Indeed, of approximately 5000 patients with CHF and CAD recruited into the Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA), 47% of the patients had chest pain presumed to be angina.[426] This is consistent with the findings of other RCTs in patients with CHF where over 20% of the recruited patients, of whom 60 – 70% had CAD, had concurrent angina.[427,428] In addition myocardial ischaemia, and hence angina, may be present even in the absence of epicardial coronary artery disease.[429] Based on our own observation from 1786 patients with CHF due to LVSD in clinical setting, 27% of those with CAD and 16% of those without had experienced angina in the week prior to their scheduled clinic visit; with 33% and 24% respectively, experienced chest pain during exertion.[430] Although the aetiology of chest pain may not be due to myocardial iscahemia, angina pectoris is a strong predictor of chest pain in patients with CHF.[431] The presence of angina does not only impact on the quality of life in patients with CHF, it also confers an increased risk of heart failure hospitalisation, non-fatal acute coronary syndrome and the need for coronary revascularisation.[426]

Conventionally, patients with LVSD due to CAD are considered for revascularisation, although whether this practice is safe or effective remains controversial since the Heart Failure Revascularisation Trial[432] (HEART) and the Surgical Treatment for Ischemic Heart Failure Trial[433] (STICH) have not shown

convincing beneficial effect from conventional revascularisation in patients with CHF due to LVSD and CAD. Therefore, alternative means of improving angina when pharmacological treatment has failed, that may also be safer and more cost-effective than revascularisation, would be welcome.

As discussed earlier in Chapter 1, enhanced external counterpulsation (EECP) is a safe and non-invasive treatment that may provide sustained improvement in angina control for patients with stable CAD.[291,340] Our own pilot study on 58 patients with refractory angina confirmed that approximately 70% of the patients experienced a sustained improvement in CCS class over 1 year.[434,435] An important observation was that some patients experience an improvement in their exercise tolerance immediately following a course of EECP and this continued to improve over the next 3 to 6 months.[435] (Figure 6.1.1) In this study, 9 patients had LVEF < 35%.

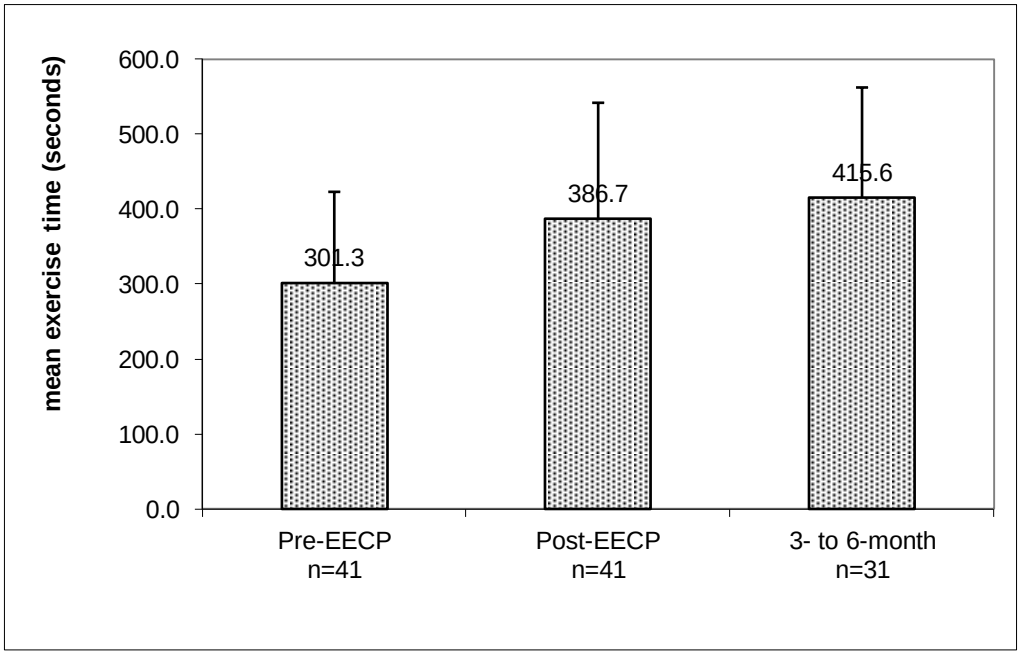


Figure 6.1.1 Exercise treadmill time before EECP, within 2 weeks post-EECP and between 3 to 6 months following EECP treatment

Based on our own observation[436] and the data from over 1400 patients in the International EECP Patient Registry (IEPR), 20% of whom had LVEF < 35%, we have also shown that the beneficial effects of EECP can be sustained for up to 3 years in the majority of the patients.[437,438] (Figure 6.1.2) In a series of 18 patients, Lawson et al has shown that the beneficial effects of EECP might be sustained for up to 5 years.[343] In a

separate series based on 91 patients in the IEPR, we have shown that EECP can improve angina control even in the majority of patients with significant coronary disease who had had prior conventional and conconmittant laser myocardial revascualrisation.[439] Of these patients, 23% had LVEF < 35% and 41% had a prior history of CHF. This makes EECP an attractive treatment for the patients with CHF due to CAD as most of these patients have extensive coronary disease.[432,433]

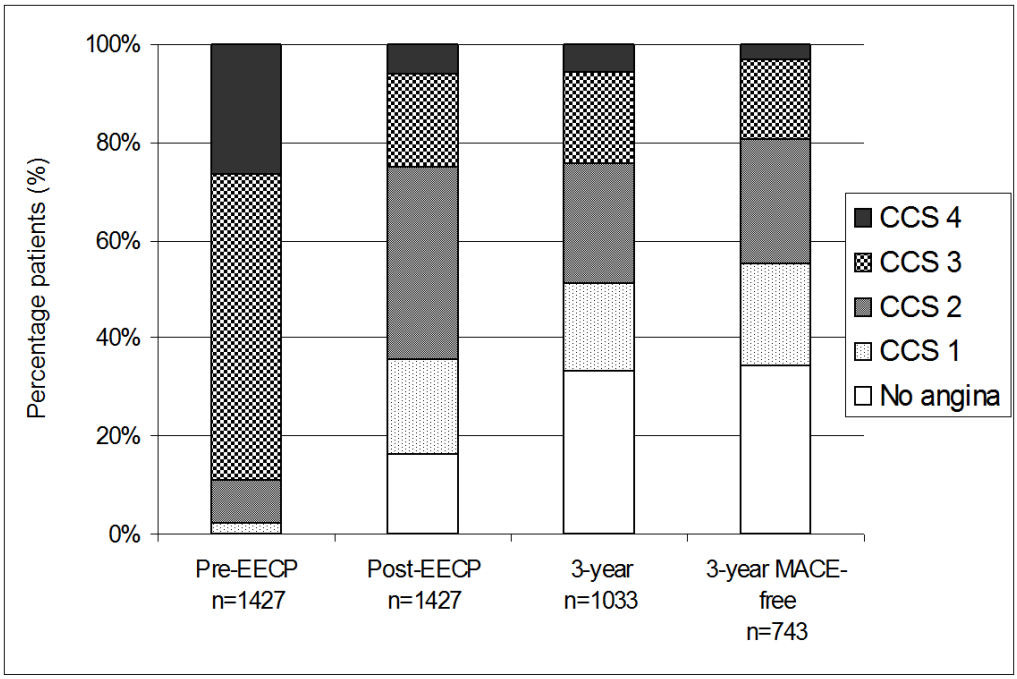


Figure 6.1.2 CCS angina grading at pre-EECP, post-EECP and 3-year follow-up including the 3-year CCS class in patients without a major adverse cardiovascular event (MACE-free CCS class)

In a group of patients with refractory angina, Lawson et al. compared the outcome of 20 patients with LVEF > 35% to 5 patients with LVEF <= 35% and found that EECP was safe and had similar efficacy in both group of patients in improving their angina control.[325] Bioimpedance measurements in that study showed an increase in cardiac power, stroke volume and cardiac index with a reduction in systemic vascular resistance in patients with LVEF <= 35%. This suggests that EECP may be beneficial in patients with LVSD and CHF.

A multicentre observational study confirmed the safety and potential benefits of EECP in CHF patients.[348] Eleven patients with idiopathic dilated cardiomyopathy and

21 patients with LVSD due to CAD with NYHA II and III functional state experienced sustained improvement in exercise tolerance, peak oxygen consumption (pVO_2) and quality of life based on Minnesota Living with Heart Failure Questionnaire (MLHFQ) for at least 6 months following a course of EECP treatment. In the Prospective Evaluation of Enhanced External Counterpulsation in Congestive Heart Failure (PEECH) trial, EECP treatment led to a sustained improvement in the exercise tolerance of 93 patients with LVEF $\leq$ 35% after 6 months follow-up when compared to non-treatment controls.[292] This was mainly observed in patients with ischaemic heart disease. However, an associated increase in pVO_2 was only observed in the pre-specified subgroup of patients who were older than 65 years of age.[297] Observational data from over 300 patients in the Phase 1 of IEPR (IEPR-1) have confirmed that the improvement in angina can be sustained for up to 2 years in the majority of patients with CHF and LVEF < 35% following EECP treatment.[347,440] More recently, an observational study of 47 patients with LVSD and CAD showed that EECP improved NYHA and quality of life in these patients with associated objective improvement in LVEF and reduction in NT-proBNP level.[305]

The quality of life in patients with CHF is significantly impaired.[441] This may be due to CHF symptoms, manifestation of other co-morbid conditions including chest pain or angina, limiting physical functioning and psychosocial issues. Hence, the management for these patients should include startegies that improve quality of life. The International EECP Patient Registry Phase 2 (IEPR-2) included Kansas City Cardiomyopathy Questionnaire (KCCQ) as part of the assessment for patients with CHF. KCCQ is a self-administered 23-item questionnaire that quantifies physical limitations, symptoms, self-efficacy, social interference and quality of life and may be more sensitive than MLHFQ and Short-form 36 (SF-36).[442] Consecutive patients in participating centres treated with EECP were registered in IEPR-2 and hence representing the real-world clinical setting. The effect of EECP on the quality of life in these patients was investigated.

6.1.2 Methods

The set up of IEPR has been previously described.[341] Consecutive patients registered in IEPR-2 between February 2003 and October 2004 with refractory angina and CHF were included in this analysis. The short-term effects of EECP on symptoms

and health status using Canadian Cardiovascular Society (CCS) angina grading, New York Heart Association (NYHA) breathlessness classification, Duke Activity Status Index[443] (DASI) and KCCQ[442] were measured at baseline and within a week following a course of EECP.

The 23-items (16 questions) in KCCQ were grouped accordingly into a few domains: physical limitations (question 1), symptoms (frequency [questions 3, 5, 7 and 9], severity [questions 4, 6 and 8] and change over time [question 2]), self-efficacy and knowledge (questions 11, 12), social interference (question 16) and quality of life (questions 13 – 15). The scale scores are transformed to a 0 to 100 range by subtracting the lowest possible scale score, dividing by the range of the scale and multiply by 100. Two summary scores can be obtained to represent functional status score (combining physical limitation and symptom domains without the symptom stability) and clinical summary score (combining functional status with quality of life and social limitation domains).

All clinical events were recorded according to IEPR set up and major adverse cardiovascular event was defined as composite of death, myocardial infarction (MI), coronary artery bypass graft surgery (CABG) and percutaneous coronary intervention (PCI).

Continuous data were compared using paired *t*-test and categorical data by Chi square test. Barker's test was used for paired comparison of CCS and NYHA. All statistical tests were two-tailed, and probability of less than 0.05 was considered significant.

6.1.3 Results

One hundred and thirty nine patients were included in this analysis. (Table 6.1.1) Of these, 79% compeleted their course of EECP. The mean treatment duration was 32±10 hours. During treatment, 6% had exacerbation of heart failure and 2% had a major cardiovascular event (death/myocardial infarction/CABG/PCI). (Table 6.1.2)

Table 6.1.1 Baseline characteristics of all patients (n=139)

Baseline characteristics	n = 139
Age (years)	69.3 ± 9.7
Men	75.5%
LVEF (%)	34.6 ± 15.8
LVEF < 35%	53.3%
Duration of coronary artery disease	12.7 ± 8.4
Multivessel coronary artery disease	94.6%
Prior myocardial infraction	82.6%
Prior PCI	62.2%
Prior CABG	69.9%
Prior PCI or CABG	88.4%
Unsuitable for revascularisation	92.6%
Prior EECP	7.3
Diabetes mellitus	53.0%
Hypertension	79.7%
Peripheral vascular disease	24.6%
Chronic kidney injury	23.8%
CABG, coronary bypass graft surgery; EECP, enhanced external counterpulsation; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention.	

Table 6.1.2 Treatment and treatment events

Treatment	
Mean treatment duration (hours)	32.2 ± 11.2
Treatment completed	79.1%
Patient discontinued	12.2%
Stopped due to event	6.5%
Events	
Skin breakdown	4.7%
Musculoskeletal pain	2.3%
Unstable angina	0.8%
Myocardial infarction	2.3%
PCI	0
CABG	0.8%
Heart failure	6.2%
TIA/stroke	0.8%
Death	0
MACE	2.2%

CABG, coronary artery bypass graft surgery; TIA, transient ischaemic attack.
Major adverse cardiovascular event (MACE) defined as composite of death, myocardial infarction, PCI and or CABG.

The medications were largely unchanged following EECP apart from a reduction in short-acting nitrate usage. (Table 6.1.3)

At baseline, 94% had CCS III/IV angina. (Figure 6.1.3) EECP improved angina by at least 1 CCS grade in 78% of the patients whilst reducing angina by 10±18 episodes/week and GTN use by 7±10 times/week (all $p<0.001$). (Figure 6.1.4) GTN was discontinued in 40% of the patients. (Table 6.1.3)

Table 6.1.3 Medications before and following EECP treatment

	Pre-EECP	Post-EECP
Anti-platelets	81.2	78.8
Warfarin	24.1	22.6
β-blockers	99.1	99.0
Calcium channel blockers	32.1	30.1
Short-acting nitrates*	71.3	48.9
Long-acting nitrates	76.8	71.7
ACEIs	52.6	52.6
ARBs	19.7	19.7
Diuretics	81.2	78.8
ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker *$p< 0.001$ comparing pre- and post-EECP		

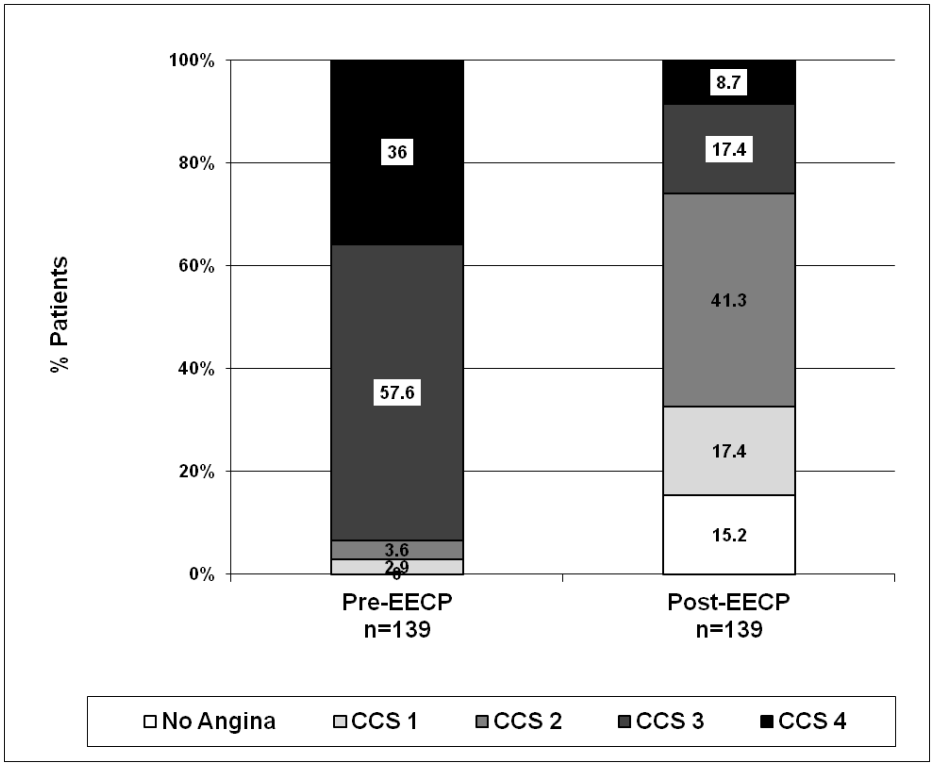


Figure 6.1.3 CCS class before and after EECP in all patients (n=139)

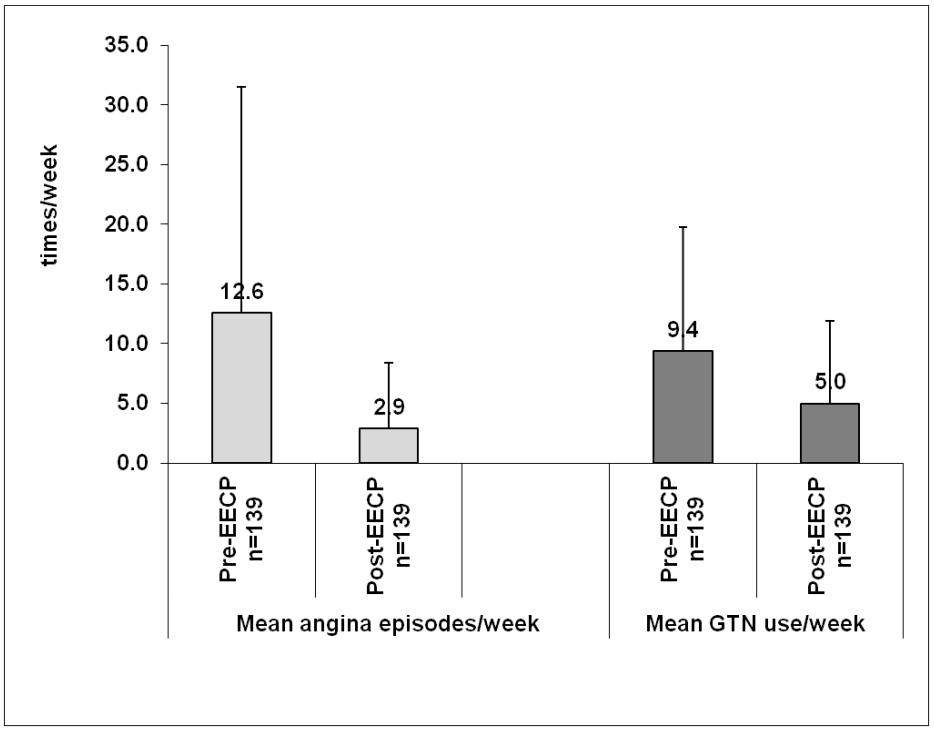


Figure 6.1.4 Weekly angina frequency and GTN use before and after EECP (n=139)

Of the 84 patients with NYHA data available, 56% had NYHA III or IV breathlessness at baseline. (Figure 6.1.5) EECP significantly improved their symptoms by at least 1 NYHA class in 44% of the patients ($p<0.001$).

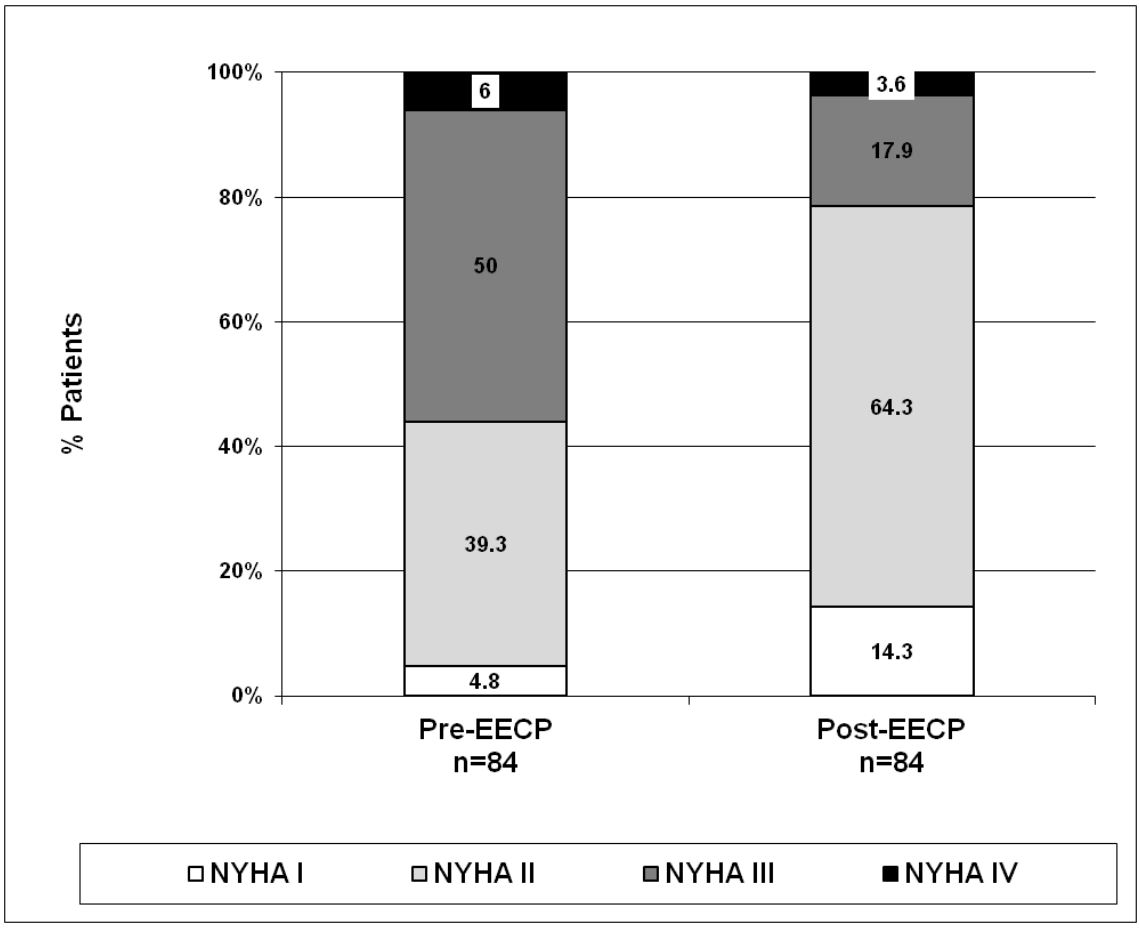


Figure 6.1.5 NYHA class of patients before and after EECP (n=84)

The DASI improved by a mean of 4.3 ± 8.4 following EECP; whilst KCCQ clinical summary improved by 10.2 ± 15.9 and KCCQ functional status by 13.9 ± 14.5, all p<0.001. (Table 6.1.4) Similar pattern and extent of improvement was observed when comparing 50 patients with LVEF > 35% and 89 patients with LVEF <= 35%.

Table 6.1.4 Duke's Activity Statatuas I ndex (DASI), Kansas City Cardiomyopathy Questionnaire (KCCQ) of patients at baseline and following EECP treatment

	Pre-EECP	Post-EECP	p
DASI	9.0 ± 8.4	13.3 ± 10.1	<0.001
KCCQ			
Physical limitation	47.5 ± 19.8	58.9 ± 19.7	<0.001
Symptom	48.6 ± 20.2	65.8 ± 21.6	<0.001
Symptom frequency	51.9 ± 23.3	68.5 ± 23.4	<0.001
Symptom severity	48.7 ± 20.3	64.8 ± 22.2	<0.001
Symptom stability	45.7 ± 22.4	64.8 ± 23.1	<0.001
Self-efficacy	82.3 ± 18.2	89.4 ± 14.2	<0.001
Social interference	45.3 ± 24.6	57.5 ± 23.1	<0.001
Quality of life	43.9 ± 25.2	59.2 ± 26.0	<0.001
KCCQ clinical summary	49.2 ± 21.6	65.1 ± 21.8	<0.001
KCCQ functional status	46.9 ± 17.7	60.7 ± 18.6	<0.001

6.1.4 Discussion

Using disease-specific KCCQ, this study shows that EECP can improve the quality of life in patients with angina and CHF within the actual clinical setting. The improvement was seen across all domains of KCCQ and also the combined clinical summary and functional status components of the questionnaire. The improvement was associated with better angina control, lesser degree of breathlessness and increased physical activity as assessed by DASI.

The prognosis and quality of life in patients with CHF has improved significantly in the last two decades with modern heart failure treatment that includes device therapy.[369,413,444] However, CHF symptoms, manifestation of other co-morbid conditions including angina, limiting physical functioning and psychosocial issues lead to poor quality of life.[441] Various psychosocial factors may, in turn, affect the course of CHF.[445] It is also unclear which components of these psychosocial factors may affect the outcome of CHF.

For example, depression is 4 – 5 times more common in CHF than general population and it affects 20 – 40% of the patients with CHF.[446] Depressed CHF patients have increased risk of hospitalisation and mortality, whilst depressive symptoms are associated with 2 – 3 folds increased mortality risk independent of the established clinical or biological prognostic factors.[447] Therefore, treatment of CHF should include intervention(s) that may improve the quality of life with or without prognostic benefit. However, intervention such as the use of sertraline, a serotonin re-uptake inhibitor, has not been proved to be useful in this setting.[448]

In addition, the DASI of the patients in this study improved following a course of EECP. DASI has been shown to be a valid tool to estimate peak oxygen uptake. It also provides an accurate assessment of functional capacity in patients with CHF.[449] DASI takes into account of the cardiac and peripheral (muscular and vascular) effects of CHF has on the patients.[450] Further, DASI is also a prognostic marker of CHF independent of the established clinical and biological predictors including B-type natriuretic peptide.[451] EECP improves the exercise tolerance of patients with CHF[292,348] and in older patients, it may improve peak oxygen uptake.[297]

In this study, over 30% of the patients were freed from angina or experienced angina only on strenuous exertion (CCS I) after completed a course of EECP. In patients with CHF and a history of angina, those with ongoing angina have a higher risk

of requiring hospitalisation for heart failure or suffering an acute coronary syndrome or acute coronary syndrome plus the need for coronary revascularisation. In 2,376 patients with CAD and LVEF < 40% undergoing cardiac catheterisation, 59% of the patients had angina despite optimal medication and a high prevalence of prior revascularisation. The presence of angina was associated with a higher risk of cardiovascular death and rehospitalisation.[452] In addition, those with persistent angina for at least 1 year may have a higher risk of major adverse cardiovascular events.[453] In the STICH study, surgical revascularisation in patients with LVSD due to CAD did not confer survival benefit but led to a modest reduction in hospitalisation for heart failure suggesting that alleviation of myocardial ischaemia may reduce morbidity in patients with CHF.[433]

EECP is non-pharmacologic, non-invasive therapy which is safe in patients with CHF and may reduce myocardial ischaemia. Therefore it may be an adjuvant treatment for patients with CHF since it has beneficial effect across all domains and components of the KCCQ as well as improving DASI and angina control. Whether EECP could alter the disease course of patient with CHF with or without angina remains to be investigated.

6.1.4.1 Limitations

This is an observational study but as consecutive patients with CHF were included in the study, it represents the experience in real-world clinical setting. The patients were referred for EECP treatment due to refractory angina and not for CHF. It is also unknown if the immediate benefit following EECP treatment may be sustained for longer period of time and further follow-up data would help to clarify this.

6.1.5 Conclusion

In this multi-centre, observational study, EECP had high patient acceptance and was effective at improving symptoms and health status in patients with both angina and heart failure. As no treatment, including revascularisation, has been shown to be safe or effective for the management of angina in patients with heart failure, EECP could be considered first-line therapy when pharmacological and device treatment has failed.

6.2 Enhanced External Counterpulsation in patient with coronary artery disease and left ventricular systolic dysfunction

6.2.1 Introduction

Ischaemic heart disease (IHD) is the commonest cause of chronic heart failure (CHF) due to left ventricular systolic dysfunction (LVSD) in industrialised countries and constitutes 50% of such cases. Despite the prognostic benefit of modern heart failure treatment, the overall survival remains poor with 20 - 30% of CHF patients die within 3 years.[369] The prognosis of patients with CHF and coronary artery disease (CAD) is worse than those without CAD.[454] This is partly related to the angiographic severity of CAD.[455] However, conventional invasive myocardial revascularisation interventions fail to improve the prognosis of these patients with little benefit to their quality of life.[432,433]

Enhanced External Counterpulsation (EECP) is a safe and effective outpatient-based non-invasive treatment for patients with symptomatic CAD who are not suitable for revascularisation.[291,435,438] It consists of ECG-gated sequential compression of lower extremities using three pairs of pneumatic cuffs applied to the calves, thighs and buttocks. These cuffs are inflated proximally in a sequential fashion during diastole and deflated simultaneously at the onset of systole. These external mechanical activities lead to diastolic augmentation and systolic unloading similar to the effect of intra-aortic balloon pumping (IABP) with additional effect on the peripheral venous system and increases venous return.[260,261] EECP increases diastolic and mean coronary pressures and flow whilst reducing systolic pressure in the central aorta and the coronary artery.[302] EECP also improves left ventricular (LV) diastolic filling, decreases end-diastolic pressure, and improves LV peak filling rate, end-diastolic volume and time-to-peak filling rate.[304] A typical course of treatment involves 35 one-hour treatment sessions over 4 to 7 weeks.

An earlier study using the hydraulic mediated device, a precursor of EECP, in patients with HF secondary to IHD has demonstrated that external counterpulsation increases cardiac output and decreases oxygen consumption without any adverse effect.[456] EECP has also been shown to be beneficial in patients with CAD and LVSD.[457] More recently, EECP has been reported to be safe and beneficial in patients with CHF leading to improvement in exercise capacity, quality of life and functional

status without any cardiovascular event at 1-week and six-month post-treatment.[348] Observational data from International EECP Patient Registry (IEPR) has also reported that EECP is a safe and effective treatment for angina in patients with CHF.[299] (28) In addition, EECP improved dobutamine-stress wall motion score in patients with CAD suggestive that it may potentially prevent stress-induced ischaemia and myocardial stunning, hence reversing LVSD.[328] In the Prospective Evaluation of Enhanced External Counterpulsation in Congestive Heart Failure (PEECH) trial, EECP treatment led to a sustained improvement in the exercise tolerance of patients with LVEF $\leq$ 35% after 6 months follow-up when compared to non-treatment control.[292] This was mainly observed in patients with underlying IHD. Although there was no associated increase in the peak oxygen uptake (pVO_2) in the overall study cohort, sustained increase in exercise tolerance and pVO_2 was observed in the pre-defined subgroup of patients older than 65 years.[297] There was also associated medium-term improvement in the subjective functional class and quality of life.[292]

The mechanisms of action of EECP remain unclear but likely to be multi-factorial. EECP enhances peripheral endothelial function[318] and may increase angiogenesis and coronary collaterals[310,324] with the improvement in myocardial perfusion at rest and during stress.[324] EECP also improve regional myocardial oxygen metabolism[458] and reduces plasma level of brain natriuretic peptides.[304] On the other hand, the effect of EECP may be attributed to peripheral training effect.[325] In addition, the possibility of placebo effect cannot be excluded.[338,339]

Cardiac magnetic resonance imaging (CMR) is the gold standard diagnostic tool with high spatial resolution to quantify the LV volumes, mass and scar extent with the ability to assess myocardial perfusion.[459] The primary aim of this study was to assess the immediate and medium-term effect of EECP on LV function in patients with IHD and LVSD using CMR. The effect of EECP on myocardial perfusion and whether the LV scar extent affects the response to EECP were investigated. The effect of EECP on exercise tolerance and quality of life of these patients was also assessed.

6.2.2 Methods

6.2.2.1 Design

This is a prospective randomised controlled study comparing patients receiving 35 one-hour EECP treatment sessions (Active) to those receiving 35 five-minute EECP treatment sessions (Control). Patients were randomised at 1:1 ratio according to minimisation method based on sex, NYHA class I/II and III/IV and LVEF < 35% using minim.exe, a free downloadable software.[460] Participating patients were assessed at baseline and within 2 weeks and at 6 months after the treatment. In addition to routine physical examination and blood and 24-hour urinary tests, transthoracic echocardiogram (TTE), cardiopulmonary exercise test (CPET) and basic, gadolinium late enhancement and rest and adenosine-stress first pass CMR were performed.

6.2.2.2 Outcome measures

The primary outcome was the improvement in global left ventricular function by a 5% point increase in LVEF measured on CMR. The secondary CMR outcomes were the improvement in the global first-pass reserve index (FPR) and the changes in LV volumes and end-diastolic mass (indexed to body surface area) and the number of viable but dysfunctional myocardial segments. Other secondary outcome measures included the change in treadmill exercise time and CPET measurements, New York Heart Association functional class (NYHA), Duke's Activity Status Index[443] (DASI) and Minnesota Living with Heart Failure Questionnaires[461] (MLHFQ).

6.2.2.3 Patients

Clinically stable CHF patients with LVEF < 50% on CMR and IHD over the age of 18 years who were able to give informed consent were included in the study. The patients were on optimal CHF medications for at least 3 months. The exclusion criteria include: previous EECP treatment, acute coronary syndrome within the past 3 months, refractory angina in whom EECP was considered to be beneficial for symptomatic relief, arrhythmias that would interfere with the triggering mechanism of the EECP treatment console and/or image acquisition of CMR, clinically significant valvular heart disease, uncontrolled hypertension (blood pressure higher than 180/100 mmHg), venous thromoboembolism or acute thrombophlebitis within the last 3 months, aortic aneurysm (diameter < 4.0 cm), primary or secondary coagulation abnormalities with INR > 2.5, pregnancy and contraindications to CMR.

6.2.2.4 EECP treatment and monitoring

The patients were treated using the model TS3 EECP treatment console in accordance to the EECP Operation Manual (Vasomedical, Inc., Westbury, New York, USA) by trained physicians or specialist nurses. A total of 35 sessions were administered over 3 to 7 weeks (weekdays only). Each session lasted 1 hour in the Active group and 5 minutes in Control group. A maximum of two sessions were given in a day with at least an hour of resting period in between the two treatment sessions.

Three pairs of pneumatic cuffs were applied to the patient's buttocks, thighs and calves. These were inflated at 80 mmHg and gradually increased to a maximum pressure of 260 mmHg within the first minute of each treatment session. The timing for cuff inflation and deflation was then adjusted to obtain an optimal diastolic augmentation (DA). DA was indicated by the ratio of peak systolic pressure to the peak augmented diastolic pressure (P) and the ratio of the area under the pressure curve during the non-augmented phase to that during the augmented phase of each cardiac cycle (A). The Control group received a full 5 minutes of treatment after an optimal DA was achieved. For the Active group, the cuff inflation timing and DA were assessed and adjusted if indicated every 15 minutes.

Every patient was examined by a doctor before and after each treatment session to ensure that they were clinically stable. Their blood pressure, heart rate, respiratory rate, pulse oximetry and bioimpedence body composition (Tanita TG410MA Body Composition Analyser, Illinois, USA) were recorded. Pulse oximetry was monitored regularly throughout the treatment session. Any adverse event associated with the treatment was documented and the treatment was discontinued if the patient was deemed unsuitable for further treatment.

6.2.2.5 Transthoracic echocardiography

TTE was performed using the Vivid Five system (Vingmed Technology, General Electric Healthcare, Wisconsin, USA) in accordance to the Guidelines from the British Society of Echocardiography. Digital images were recorded and analysed using EchoPac 6.4.1 alias (General Electric Healthcare, Wisconsin, USA).

6.2.2.6 Spirometry and cardiopulmonary exercise test (CPEx)

Spirometry was performed in upright sitting position prior to CPEx. The force expiratory volume (FEV_1), functional vital capacity (FVC), peak expiratory flow rate

(PEFR) were measured and the percentage age-predicted FEV1 and FVC were calculated.

CPEx was conducted by a well-trained technician under the direct supervision of a medical doctor in a safe environment equipped with all essential cardiopulmonary resuscitation equipment. The patients were instructed not to eat or smoke for 3 hours prior to the test. No unusual effort was performed for at least 12 hours before the test. All patients underwent a treadmill-based symptom-limited CPET with metabolic gas exchange (Jaeger Oxycon Delta, Viasys, USA) using an incremental Modified Naughton protocol. Each patient had a practice test within 2 weeks of the baseline assessment. The pVO_2 was calculated as the average VO_2 for the last 30 seconds of exercise. The ventilation and carbon dioxide production (VE/VCO_2) slope (full) was calculated using linear regression by analysing breath-by-breath values obtained throughout the full test.[462] The anaerobic threshold (AT) was calculated using the VO_2/VCO_2 method.[463] The VE/VCO_2 slope at AT was also calculated. The peak respiratory exchange ratio (RER), (VCO_2/VO_2) gave an indication of the patient's exercise effort.[464]

6.2.2.7 Cardiac magnetic resonance imaging

Patients underwent CMR on a 1.5-Tesla scanner (Signa CVi, GE Medical Systems, Wisconsin, USA) using ECG-triggered breath-hold gradient-echo in steady-state acquisition (FIESTA) imaging. The first-pass perfusion gadolinium-enhanced CMR was performed using a fast inversion recovery gradient-echo sequence[465] in a multislice fashion with six interleaved short-axis sections. Patients first underwent first-pass perfusion CMR imaging at rest. A gadolinium-based contrast agent (gadopentetate dimeglumine or gadoteridol, Omniscan, GE, Wisconsin, USA) was administered intravenously (0.05 mmol/kg) using a power injector. Patients were instructed to hold their breath for as long as possible during image acquisition. After 15 minutes to allow for the clearance of the initial contrast injection, adenosine will be administered intravenously at 140 μg/kg/min. After 3 minutes of adenosine infusion, another gadolinium first-pass perfusion images were acquired. The adenosine infusion was only discontinued at the completion of image acquisition.

Another dose of gadolinium (1 mmol/kg) was administered immediately upon completion of the adenosine-stress first pass imaging. The late gadolinium enhancement (LGE) image acquisition was performed 10 – 15 mins later using a segmented

inversion-recovery fast gradient echo sequence.[466] These images were acquired in 3 identical short-axis views starting with a basal slice 1 cm below the aortic outflow tract and finishing before the apical slices to avoid the partial volume effect.[467] These delayed-image prescriptions had the same slice thickness and spacing as the baseline short-axis cine images.

All image analyses were performed by an experienced observer blinded from the patient treatment group using an off-line workstation and MRI-MASS software (Medis, Leiden, The Netherlands). Patient's heart rate during CMR was documented. Cine images were used to calculate LV end-diastolic volume (LVEDV), end-systolic volume (LVESV) and myocardial mass at end-diastolic phase (LVEDM). The LV ejection fraction (LVEF), stroke volume (SV) and cardiac output were calculated. These were indexed according to patient's body surface area (BSA) which was calculated using Mosteller method[468].

The analysis of LV regional function was performed in a 17-segment model[469] by the modified centreline method.[470] In each of 3 short axis slices (basal, mid-cavity and apical slices), wall thickening was calculated for 100 centreline chords which will then be reordered into 6 proportionally sized regions. The position of reference point (chord 1) was identified manually at the end-diastolic and end-systolic phases of each slice. This was the point where the endocardial surface of the posterior right ventricular free wall met the interventricular septum. The apex could not be assessed using this method. For each of the resulting 16 segments, the mean, standard deviation (SD), minimum and maximum values of wall thickening were computed. Segments with systolic wall thickening within the range of mean ± 2 SDs of reference values were considered normal.[471] The wall motion in each or the 17 segments was assessed independent to the delayed enhancement images. The wall motion was classified as normal or dysfunctional (hypokinetic, dyskinetic or akinetic) visually in combination with wall thickening data.[472] The thickening and motion of the apex was assessed visually.

The LGE images were analysed based on the same 17-segment model. The hyperenhanced segments will be defined as image intensities > 2 SDs above the mean of the normal regions and the number of hyperenhanced segments was recorded. In addition, the scar area on each of the short-axis images (apex excluded) was be assessed quantitatively by manually contouring the hyperenhanced areas. The scar volume in

each patient was calculated as total scar area multiplied by the section thickness and the specific gravity of the myocardium (assumed to be 1.05 g/ml).[473] The transmurality scar thickness of each segment including the apex was graded semi-quantitatively using the following grade: no delayed enhancement, 1 – 25%, 26 – 50%, 51 – 75% and > 75% of wall thickness.[472]

The gadolinium first-pass images were analysed by measuring the signal intensity of myocardium using the time-intensity analysis module of the software based on the same 17-segment model (apex excluded). The maximal signal intensity in each slice was defined for 100 centreline chords which were then reordered into 6 proportionally sized segments. For each segment, the mean, SD, minimum and maximum values of signal intensity were computed. Segments with signal intensity of 2 SDs below mean in normal myocardium will be determined to represent areas with reduced perfusion. The number of segments with perfusion deficit at rest and under stress will was recorded. The rate of change in signal intensity for each segment was calculated from the signal intensity-time curves of the rest and stress perfusion images. The segmental first-pass reserve index (FPRI) was then calculated as the ratio of the signal intensity change rate at stress to that of at rest. Each patient's global FPR was calculated as the mean of the segmental FPRI values.[474] The global FPR was assumed to represent an estimation of the global myocardial perfusion index.

As only dysfunctional myocardial segments with scar <= 50% of the myocardial wall thickness are likely to recover in wall motion following revascularisation[475,476], segments with scar > 50% of wall thickness were not included for wall motion and FPRI comparison before and after EECP treatment and the comparison between treatment groups.

The high inter- and intra-observer agreement in image analysis within the department have been documented.[472,477]

6.2.2.8 Laboratory tests

Blood was taken and biochemical profile, albumin, full blood count, high sensitive c-reactive protein (hs-CRP), NT-proBNP and high-sensitivity troponin T (hs-TnT) were measured in our local hospital laboratory. Additional blood samples were collected into 3.2% sodium citrated (Greiner Bio-One GmbH, Austria). These samples were centrifuged immediately at 3000 rpm for 15 minutes in a refrigerated centrifuge at

4 °C and the plasma was stored at -80 °C. These plasma samples were used in the assays of H-FABP (HyCult Biotechnology, Uden, The Netherlands), D-dimer (TintElize® D-dimer, Trinity Biotech, Ireland), fibrinogen (AssayPro, Universal Biologicals, UK) and vWF activity (REAADS, Corgenix, UK) in our own research laboratory using commercially available assay kits. (Refer to Appendix for full assay information) The 24-hour urine samples were analysed in local hospital laboratory.

6.2.2.9 Statistics

Based on the primary outcome measure of a 5% point increase in LVEF and a SD of 5%, 22 patients were required in each group in order to provide a 90% statistical power (5% significance, two-tailed). The study planned to recruit 60 patients in order to allow for a 30% dropout rate.

For continuous data, paired comparison between pre-EECP and follow-up data within Active and Control groups and unpaired comparison between the two groups were performed using paired and unpaired Student's *t* test respectively. Between group comparison of categorical data were performed using Chi square or Fisher's exact tests depending on the number of patients in each group. Paired categorical data were compared using McNemar's test. All statistical tests were two-tailed, and probability of less than 0.05 will be considered significant. The analyses were performed on a personal computer using Statistical Package for Social Sciences 13.0 (SPSS, IBM, Chicago, USA).

6.2.3 Results

The enrolment was slow as many patients unwilling to participate in view of the long treatment schedule. The enrolment period began when cardiac resynchronisation therapy began to be an established treatment for suitable patients and this precluded them from having CMR and taking part in the study. Hence only 17 patients were enrolled, 10 were assigned to Active and 7 to Control groups. However, 2 Active patients dropped out due to treatment events. A 54-year old man had sudden onset left facial parasthesia 5 minutes into the first treatment session. This resolved within 2 days and was later diagnosed as a cerebral vascular event by a stroke physician clinically without any infarct lesion on the cerebral computed tomography. Another 72-year old man dropped out after the first treatment session due to aggravation of the back pain

which resolved two weeks later. A 51-year old man assigned to Control group stopped after 8 treatment sessions due to his work schedule. These patients withdrew their consent to return for follow-up. Therefore, only data from 8 Active and 6 Control patients were available for analysis.

6.2.3.1 Baseline characteristics and treatment variables

The baseline characteristics were similar in the Active and Control patients as shown in Table 6.2.1.

All the patients completed 35 sessions of treatment course without any treatment event. The Active patients took longer than the Controls to complete their treatment course. However, both groups were similar in the treatment observations and diastolic augmentation (Table 6.2.2). These treatment variables did not change when comparing the first to the last treatment sessions in both patient groups (Table 6.2.2).

6.2.3.2 Cardiac magnetic resonance imaging and Echocardiography

There was no difference in the volumetric analysis of CMR images in both the Controls and Active patients at baseline, post-EECP and 6-month follow-up. (Table 6.2.3) Following EECP treatment, both groups of patients had a reduction in LVEF which was persistent in the Controls whilst that of the active treatment group returned to baseline level. (Table 6.2.3) Similar trend was observed in the corresponding decrease in LVEDV, LVESV and CI especially in those patients who received active treatment.

On LGE image analysis, the mean number of LV segments with and without LGE was similar in both groups of patients. (Table 6.2.3) Overall, 12/102 (11.8%) segments in the Controls and 21/136 (15.4%) in the Active patients had > 50% wall thickness with LGE and were excluded from subsequent analysis of wall motion due a lack of potential for functional recovery (mean number of viable segments was 15.0 ± 1.9 in Controls vs 14.4 ± 2.7 in Active patients, $p = 0.64$). Overall, 14/102 (13.7%) segments in the Controls and 24/136 (17.6%) segments in the Active patients were excluded for perfusion analysis due to the lack of potential for functional recovery or unavailability of perfusion data in the apical segments (mean number of segments available for perfusion analysis was 14.7 ± 1.5 segments in the Controls vs 14.1 ± 2.4 segments in the Active patients, $p = 0.63$). The mean number of viable but dysfunctional segment and ischaemic segment were similar in both groups of patients at baseline,

post-EECP and 6-month follow-up. The FPRI was also similar in both groups of patients at baseline, post-EECP and follow-up. (Table 6.2.3)

The echocardiographic measurements were similar in both the Control and Active groups at baseline, post-EECP and 6-month follow-up. These measurements did not changed significantly following EECP treatment or during follow-up in both groups of patient except a gradual increase in LV end-diastolic volume (LVEDV) in the Active patients. (Table 6.2.3)

When all the CMR segments in each group of patients were analysed together, there were more viable but dysfunctional segments in the Active patients than the Controls. (Table 6.2.4) However, the distribution of scar extent, the proportion of viable but ischaemic segments and the FPRI in both patient groups were similar. (Table 6.2.4) The FPRI in the active treatment patients improved following EECP and continued to do so at 6-month follow-up with an associated decrease in the proportion of dysfunctional and ischaemic segments by 6 months. In contrast, the FPRI in the Controls did not changed following EECP but worsened at 6-month follow-up with associated increase in the proportion of dysfunctional segments.

6.2.3.3 Cardiopulmonary exercise (CPEx) and spirometry

The FEV_1, FVC, PEFR and percentage age-predicted FEV_1 and percentage age-predicted FVC were similar in both groups at baseline, post-EECP and 6-month follow-up. In both groups, these variables did not change significantly following EECP or during 6-month follow-up except a short-term reduction in FVC of the controls and a minor improvement in percentage age-predicted FEV_1 in the Active patients. (Table 6.2.5)

At baseline, the CPEx variables were similar in both groups except a higher resting diastolic pressure in the Controls. (Table 6.2.5) The Controls had an increase in exercise time post-EECP with corresponding reduction in the VE/VCO_2 slope at AT but these were not sustained at 6-month follow-up. The pVO_2 in the Controls reduced marginally post-EECP before returning to the baseline level at 6-month follow-up. On the other hand, the patients in the Active group showed an increase in exercise time following EECP treatment and this continued to increase over the 6 months follow-up period but without any change in the pVO_2 and VE/VCO_2 slope. (Table 6.2.5)

6.2.3.4 Blood pressure, pulse rate and body composition by bioimpedance

Both the Control and Active groups were similar in blood pressure, pulse rate, body weight and bioimpedence-estimated body fat and water content at baseline, post-EECP and during 6-month follow-up. In both groups, all the measurements did not change following EECP treatment and during 6-month follow-up except a reduction in pulse rate of the controls post-EECP but this was not sustained at 6-month follow-up. (Table 6.2.6)

6.2.3.5 Laboratory tests

The blood and 24-hour urinary test results were similar in both groups of patient at baseline. (Table 6.2.7) The majority of the blood test results did not change following EECP treatment and at 6-month follow-up except a non-sustained reduction in H-FABP level in the Active patients and fibrinogen in the Controls. All of the 24-hour urinary test results did not changed in the Controls following EECP treatment and at 6-month follow-up. However, the urinary creatinine and creatinine clearance decreased in those patients who received active treatment at 6-month follow-up. (Table 6.2.7)

6.2.3.6 Symptoms, DASI and MLHFQ

The NYHA, CCS, weekly angina frequency and weekly GTN use were similar between the two groups at baseline, post-EECP and after 6 months. (Figure 6.2.1, Figure 6.2.2, Figure 6.2.3 and Figure 6.2.4). In the Active group, the weekly angina frequency reduced from 5.0 ± 6.0 to 1.3 ± 2.4 episodes/week ($p=0.048$) and remained low at 2.5 ± 4.8 episodes/week ($p=0.137$, when compared to angina frequency at baseline). (Figure 6.2.3) The NYHA, CCS, and GTN use in both groups and the weekly angina frequency in the Controls did not change post-EECP and during 6-month follow-up.

The DASI and MLHFQ scores were similar in both groups of patients at baseline, post-EECP and 6-month follow-up. (Table 6.2.8) The DASI score did not change following EECP treatment and during follow-up in both groups of patients. The total, physical component and emotional component of MLHFQ scores improved following treatment in the Controls but remained unchanged in the Active patients.

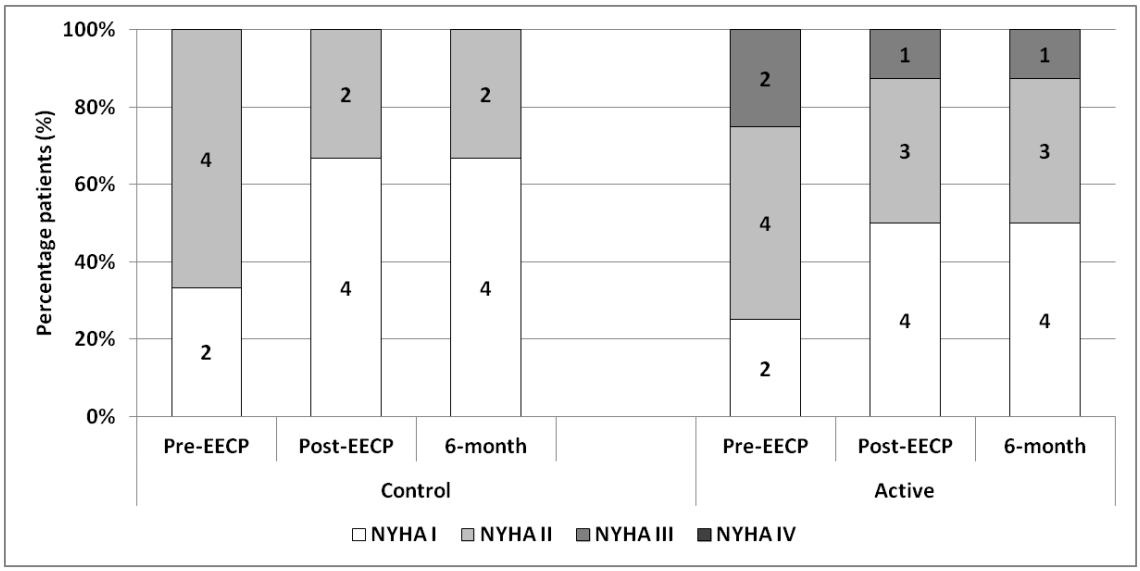


Figure 6.2.1 NYHA class of Controls and patients who received active EECP

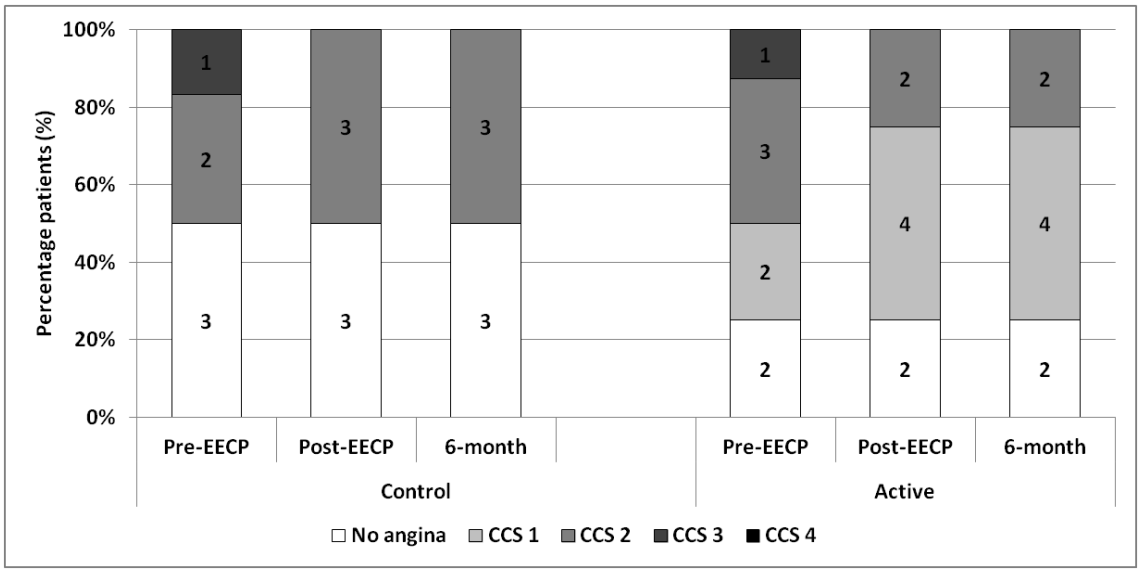


Figure 6.2.2 CCS class of Controls and patients who received active EECP

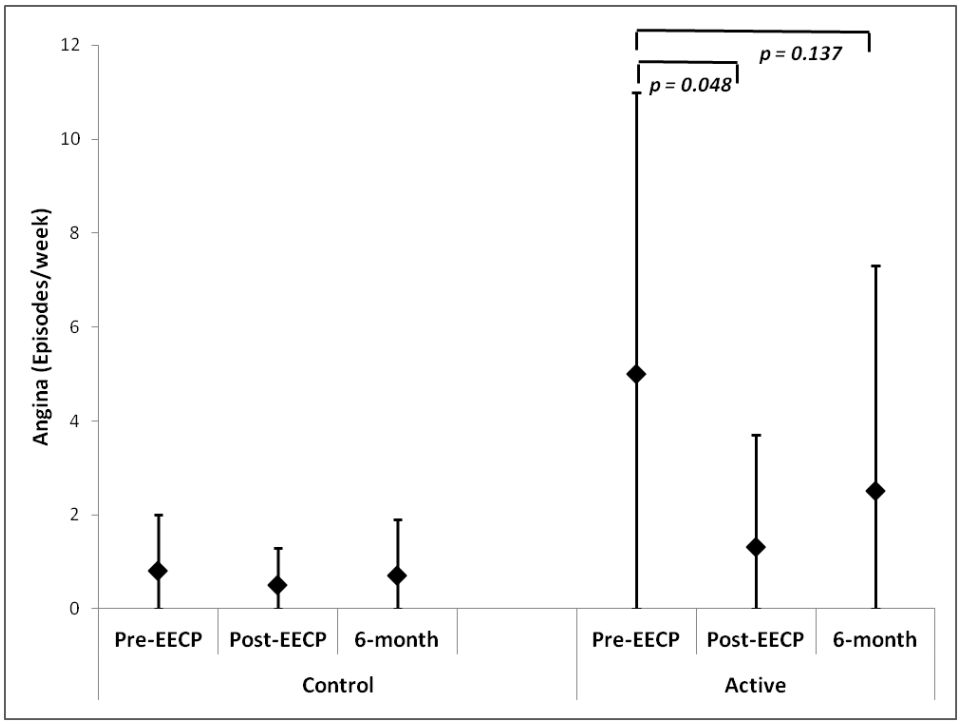


Figure 6.2.3 Weekly angina episodes in Controls and patients who received active EECP

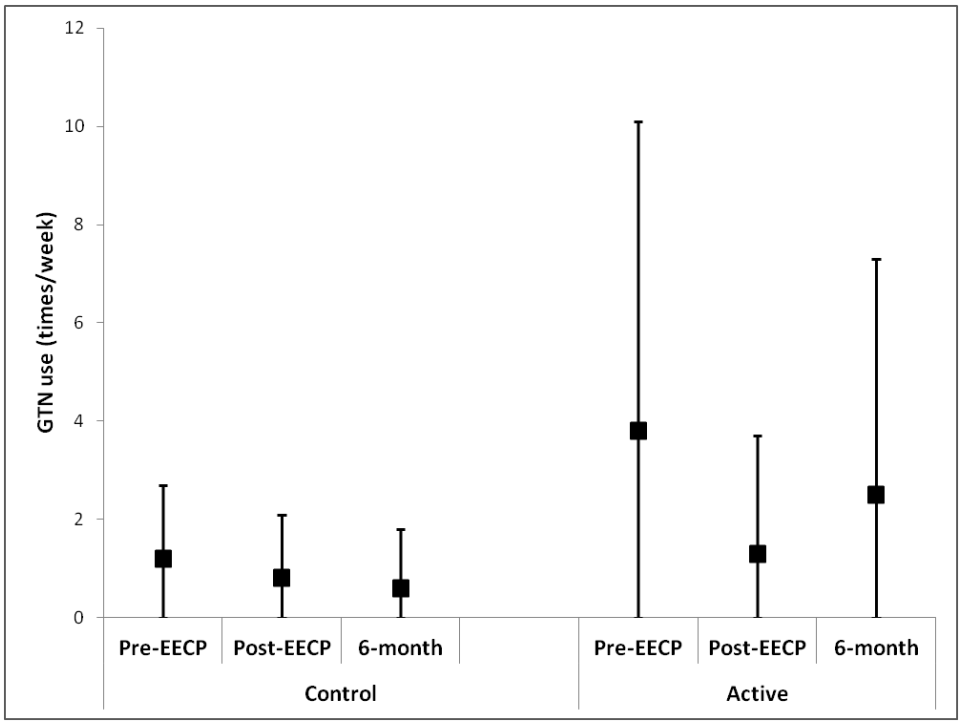


Figure 6.2.4 Weekly GTN use in Controls and patients who received active EECP

Table 6.2.1 Baseline characteristics of all patients

	Control n = 6	Active n =8	*p*
Age (years)	58.4 ± 8.9	63.3 ± 7.9	0.121
Man	6 (100)	7 (87.5)	0.369
BMI (kg/m^2)	30.9 ± 8.3	30.1 ± 5.4	0.886
BSA (m^2)	2.1 ± 0.4	2.0 ± 0.2	0.778
Length of IHD (years)	10.2 ± 6.3	9.6 ± 3.3	0.477
Previous MI	6 (100)	8 (100)	--
Previous PCI	1 (16.7)	3 (37.5)	0.406
Previous CABG	3 (50)	3 (37.5)	0.529
Diabetes	1 (16.7)	3 (37.5)	0.528
Hypertension	2 (33.4)	6 (75.0)	0.156
Atrial fibrillation	1 (16.7)	2 (25.0)	0.615
Previous venous thromboembolism	0	1 (12.5)	0.571
Medications			
ACEI/ARB	6 (100)	8 (100)	--
Beta-blocker	6 (100)	8 (100)	--
Aldosterone receptor antagonist	0	3 (37.5)	0.209
Loop diuretic	3 (50)	4 (50)	1.000
Anti-platelet	5 (83.3)	7 (87.5)	1.000
Statin	6 (100)	8 (100)	1.000
Long acting nitrate/Nicorandil	1 (16.7)	3 (37.5)	0.580
Haemoglobin (g/dL)	14.6 ± 1.0	13.9 ± 1.8	0.365
Glomerular filtration rate ($ml/min/1.73m^2$)	71.8 ± 10.3	66.0 ± 18.5	0.477
Sodium (mmol/L)	140 ± 1	139 ± 2	0.739
Albumin (g/dL)	39 ± 1	41 ± 2	0.069
Spirometry			
FEV1 (% predicted)	93 ± 15	74 ± 33	0.366
FVC (% predicted)	132 ± 35	95 ± 38	0.156
PEFR (% predicted)	67 ± 21	59 ± 33	0.699
CMR			
LVEF (%)	40.9 ± 13	30.0 ± 17	0.208

LVEDVi (ml/m^2)	119.3 ± 43.7	141.6 ± 52.9	0.421
LVESVi (ml/m^2)	73.8 ± 40.2	103.7 ± 38.2	0.278
LVEDMi (g/m^2)	81.4 ± 17.0	81.5 ± 19.8	0.997

ACEI/ARB, angiotensin converting enzyme inhibitor or angiotensin receptor blocker; BMI, body mass index; BSA, body surface area; CABG, coronary artery bypass graft surgery; FEV, forced expiratory volume; FVC, functional vital capacity; IHD, ischaemic heart disease; LVEDMi, indexed left ventricular end-diastolic mass; LVEDVi, indexed left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESVi, indexed left ventricular end-systolic volume; MI, myocardial infarction; PEFR, peak expiratory flow rate; PCI, percutaneous coronary intervention

Table 6.2.2 EECP treatment variables

	Control		Active		*p*
Mean session duration (s)	6.8 ± 0.4		60 ± 0		<0.001
Mean treatment period (days)	28 ± 5		35 ± 4		0.038
	Session 1	Session 35	Session 1	Session 35	
Heart rate (bpm)	62 ± 11	58 ± 9	68 ± 1	67 ± 11	ns
Systolic BP (mmHg)	132 ± 20	127 ± 14	121 ± 13	116 ± 13	ns
Diastolic BP (mmHg)	76 ± 7	76 ± 9	70 ± 5	66 ± 5	ns
Saturation (%)	97 ± 1	98 ± 1	99 ± 1	98 ± 2	ns
Diastolic augmentation					
P	1.2 ± 0.4	1.3 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	ns
A	1.5 ± 0.6	2.0 ± 1.0	1.2 ± 0.3	1.3 ± 0.3	ns
Mean P	1.3 ± 0.3		1.0 ± 0.3		0.474
Mean A	1.8 ± 0.8		1.3 ± 0.5		0.199
Peak P	1.7 ± 0.6		1.4 ± 0.4		0.560
Peak A	2.9 ± 1.3		2.1 ± 0.8		0.217

Data are shown as mean ± standard deviation

ns, There was no statistically significant difference found when comparing the values of session 1 to those of session 35 within the Control group and the Active group. There was also no statistically significant difference found between the Control and Active patients on all the variables in Session 1 and 35.

BP, blood pressure

Table 6.2.3 Findings on transthoracic echocardiography and cardiac MRI at baseline, post-EECP and 6-month follow-up

	Control n = 6	Active n =8	*p*
Echocardiogram:			
LVEDD (cm)			
Baseline	6.0 ± 0.8	6.2 ± 0.7	0.477
Post-EECP	6.0 ± 0.8	6.3 ± 0.7	0.452
6-month	6.0 ± 0.7	6.7 ± 0.8	0.121
LVESD (cm)			
Baseline	5.1 ± 0.8	5.6 ± 0.6	0.203
Post-EECP	5.0 ± 0.9	5.4 ± 0.9	0.505
6-month	5.1 ± 0.9	5.9 ± 1.0	0.167
LVEDV (mls)			
Baseline	214.4 ± 81.3	237.7 ± 103.5*	0.657
Post-EECP	225.3 ± 119.7	248.7 ± 88.7	0.681
6-month	232.1 ± 82.9	252.9 ± 68.8*	0.618
LVESV (mls)			
Baseline	137.7 ± 66.9	170.8 ± 93.5	0.476
Post-EECP	142.4 ± 74.8	168.6 ± 74.5	0.526
6-month	145.0 ± 49.6	179.6 ± 61.1	0.280
LVEF (%)			
Baseline	32.8 ± 9.0	30.0 ± 7.6	0.535
Post-EECP	33.5 ± 5.4	31.0 ± 6.3	0.477
6-month	34.2 ± 5.1	29.9 ± 5.4	0.159
Cardiac MRI			
Heart rate (bpm)			
Baseline	63.3 ± 11.3	71.3 ± 11.5*	0.224
Post-EECP	58.2 ± 5.7	71.0 ± 13.0	**0.045**
6-month	61.3 ± 6.9	66.6 ± 10.0*	0.290
LVEDV (mls)			
Baseline	238.1 ± 69.1	278.4 ± 85.2	0.363
Post-EECP	237.3 ± 71.4	260.6 ± 102.0	0.642
6-month	247.6 ± 85.2	261.4 ± 100.8	0.793

LVESV (mls)			
Baseline	143.4 ± 64.9	201.4 ± 91.6	0.212
Post-EECP	150.5 ± 69.9	197.7 ± 96.0	0.330
6-month	156.3 ± 76.0	189.7 ± 94.6	0.492
LVEDV index (ml/m^2)			
Baseline	119.4 ± 43.7	141.6 ± 52.9	0.421
Post-EECP	118.6 ± 43.7	133.2 ± 61.5	0.630
6-month	125.0 ± 53.8	133.7 ± 60.7	0.787
LVESV index (ml/m^2)			
Baseline	73.8 ± 40.2	103.7 ± 53.8	0.278
Post-EECP	77.6 ± 43.0	101.8 ± 56.2	0.398
6-month	81.0 ± 47.4	97.7 ± 54.7	0.563
LVEF (%)			
Baseline	40.9 ± 13.0*	30.0 ± 16.6	0.208
Post-EECP	37.3 ± 16.0*	26.4 ± 13.9†	0.194
6-month	38.1 ± 13.1*	29.7 ± 13.0†	0.258
Stroke volume (mls)			
Baseline	94.7 ± 28.8	76.9 ± 32.4	0.310
Post-EECP	86.7 ± 36.3	62.9 ± 25.3	0.171
6-month	91.3 ± 30.8	71.6 ± 22.4	0.189
Cardiac output (L/min)			
Baseline	5.9 ± 1.8	5.4 ± 2.0*	0.584
Post-EECP	5.1 ± 2.2	4.3 ± 1.2*	0.379
6-month	5.5 ± 1.7	4.6 ± 1.1	0.269
Cardiac index (L/min/m^2)			
Baseline	2.9 ± 6.7	2.6 ± 0.8*	0.597
Post-EECP	2.4 ± 0.8	2.1 ± 0.6*	0.475
6-month	2.7 ± 0.7	2.3 ± 0.6	0.334
LV mass index (g/m^2)			
Baseline	81.4 ± 17.0	81.5 ± 20.0*	0.789
Post-EECP	81.8 ± 20.9	77.7 ± 18.7†	0.565
6-month	84.1 ± 16.6	87.8 ± 20.9*†	0.959
Mean No. of segment with LGE	9.2 ± 3.1	9.1 ± 1.7	0.975

None	5.8 ± 4.2	5.3 ± 2.2	0.739
<= 50% wall thickness	2.0 ± 1.9	2.6 ± 2.7	0.640
> 50% wall thickness			
Mean No. of viable segment	15.0 ± 1.9	14.4 ± 2.7	0.640
Mean No. of viable but dysfunctional segment			
Baseline	9.0 ± 4.9	10.8 ± 4.5	0.499
Post-EECP	8.8 ± 6.1†	10.1 ± 4.3	0.651
6-month	10.7 ± 5.2†	9.5 ± 4.5	0.662
Mean No. of segment for FPRI analysis	14.7 ± 1.5	14.1 ± 2.4	0.633
Mean FPRI			
Baseline	0.92 ± 0.45	0.75 ± 0.37	0.465
Post-EECP	0.92 ± 0.35	0.77 ± 0.17	0.312
6-month	0.87 ± 0.30	0.90 ± 0.31	0.851
Mean No. of ischaemic segment	10.2 ± 3.8	12.0 ± 4.6	0.444
Baseline	10.5 ± 3.4	11.6 ± 2.9	0.518
Post-EECP	10.8 ± 3.2	10.6 ± 4.6	0.926
6-month			

* $p <$ 0.05 comparing the measurement at post-EECP or 6-month follow-up to that of baseline; † $p < 0.05$ comparing measurement at post-EECP and 6-month follow-up.

FPRI, first-pass reserve index; LGE; late gadolinium enhancement; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVEDV, left ventricular end diastolic volume; LVESD, left ventricular end systolic volume;

Table 6.2.4 CMR data analysed by left ventricular segments. The 6 controls had 90 viable from a total of 102 segments; whilst the 8 patients in the Active group had 115 viable from a total of 136 segments

	Control n = 90	Active n = 115	*p*
Total No. of segments	102	136	--
No. of segments with LGE			
None	56 (54.9%)	37 (53.7%)	0.352
<= 25%	17 (16.7%)	14 (10.3%)	
26 – 50%	17 (16.7%)	28 (20.6%)	
51 – 75%	9 (8.8%)	11 (8.1%)	
> 75%	3 (2.9%)	10 (7.4%)	
No. of viable and dysfunctional segments			
Baseline	53 (58.9%)*	86 (74.8%)*	**0.016**
Post-EECP	53 (58.9%)†	81 (70.4%)	0.085
6-month	64 (71.1%)*†	77 (67.0%)*	0.524
No. of viable and ischaemic segments‡			
Baseline	60 (68.2%)	96 (85.7%)*	**0.003**
Post-EECP	63 (71.6%)	93 (83.0%)	0.052
6-month	65 (73.9%)	85 (75.9%)*	0.742
FPRI‡			
Baseline	0.94 ± 0.72	0.74 ± 0.57*	**0.027**
Post-EECP	0.93 ± 0.53†	0.77 ± 0.47†	**0.017**
6-month	0.88 ± 0.49†	0.89 ± 0.56*†	0.940

* $p < 0.05$ comparing the measurement at post-EECP or 6-month follow-up to that of baseline; † $p < 0.05$ comparing measurement at post-EECP and 6-month follow-up.
‡ Apex was excluded from analysis and therefore 88 and 112 segments in the control and active treatment group respectively were included in the analysis.
FPRI, first-pass reserve index; LGE, late gadolinium enhancement.

Table 6.2.5 Spirometry and cardiopulmonary exercise test results at baseline, post-EECP and 6-month follow-up

	Control n = 6	Active n =8	*p*
Spirometry:			
FEV_1 (L)			
Baseline	2.9 ± 0.7	2.5 ± 0.6	0.338
Post-EECP	2.8 ± 0.4	2.5 ± 0.6	0.278
6-month	2.6 ± 0.2	2.6 ± 0.6	0.919
FVC (L)			
Baseline	5.0 ± 1.2*	4.1 ± 0.9	0.092
Post-EECP	4.3 ± 0.8*	4.3 ± 1.1	0.832
6-month	4.3 ± 0.9	4.5 ± 1.2	0.847
Percentage age-predicted FEV1 (%)			
Baseline	92.6 ± 14.7	73.7 ± 32.8*	0.217
Post-EECP	91.5 ± 18.7	72.8 ± 33.6	0.246
6-month	86.9 ± 21.5	77.4 ± 37.6*	0.594
Percentage age-predicted FVC (%)			
Baseline	131.7 ± 34.7*	95.2 ± 37.6	0.088
Post-EECP	109.4 ± 32.7*	100.2 ± 42.1	0.664
6-month	115.6 ± 38.0	105.6 ± 43.8	0.665
PEFR (L/min)			
Baseline	5.5 ± 1.9	5.6 ± 2.4	0.953
Post-EECP	5.3 ± 2.4	4.7 ± 2.8	0.247
6-month	5.4 ± 1.5	5.6 ± 2.9	0.899
Cardiopulmonary exercise			
Exercise time (secs)			
Baseline	697.5 ±	587.6 ±	0.514
Post-EECP	355.5*	257.9*	0.334
6-month	773.8 ±	623.9 ± 235.0	0.585
	324.2*	632.4 ±	
	706.5 ± 257.1	235.6*	
Resting heart rate (bpm)			
Baseline	64.7 ± 11.6	72.1 ± 10.0	0.223

Post-EECP	61.7 ± 10.7	75.1 ± 21.1	0.180
6-month	68.0 ± 17.7	71.3 ± 9.1	0.661
Resting systolic blood pressure (mmHg)			
Baseline	128.2 ± 14.6	108.0 ± 20.3	0.062
Post-EECP	125.3 ± 15.3	110.1 ± 19.9	0.146
6-month	119.2 ± 18.1	106.6 ± 19.2	0.240
Resting diastolic blood pressure (mmHg)			
Baseline	81.7 ± 8.7	65.9 ± 9.5	**0.008**
Post-EECP	79.7 ± 11.7	67.9 ± 9.6	0.060
6-month	78.2 ± 12.1	67.8 ± 5.8	0.053
Peak heart rate (bpm)			
Baseline	112.7 ± 22.6	113.5 ± 20.6*	0.944
Post-EECP	110.7 ± 15.9	122.0 ± 24.2*	0.341
6-month	113.8 ± 26.2	121.1 ± 22.8	0.589
Peak systolic blood pressure (mmHg)			
Baseline	163.2 ± 16.7	147.9 ± 33.6	0.329
Post-EECP	163.7 ± 20.1	146.6 ± 31.9	0.275
6-month	155.7 ± 30.8	144.6 ± 24.6	0.469
Peak Diastolic blood pressure (mmHg)			
Baseline	100.8 ± 28.0	78.6 ± 11.0	0.062
Post-EECP	90.5 ± 29.4	91.0 ± 28.9	0.975
6-month	80.3 ± 18.9	80.4 ± 15.7	0.996
Peak RER			
Baseline	1.2 ± 0.2	1.0 ± 0.1	0.134
Post-EECP	1.2 ± 0.1†	1.1 ± 0.1	**0.039**
6-month	1.1 ± 0.1†	1.0 ± 0.1	0.334
Anaerobic threshold, AT (mL/kg/min)			
Baseline	13.8 ± 1.8	12.0 ± 4.0	0.331
Post-EECP	13.6 ± 2.0	13.2 ± 3.4	0.792
6-month	14.8 ± 2.1	13.4 ± 3.5	0.386
pVO_2 (ml/kg/min)			
Baseline	18.9 ± 5.0	16.6 ± 4.3	0.386
Post-EECP	18.6 ± 2.8†	17.5 ± 5.4	0.655
6-month	21.7 ± 3.1†	17.5 ± 5.1	0.099

VE/VCO_2 slope (full)			
Baseline	31.7 ± 5.9	32.1 ± 5.8	0.901
Post-EECP	30.0 ± 5.7	31.7 ± 6.3	0.616
6-month	30.7 ± 4.0	32.1 ± 7.4	0.674
VE/VCO_2 slope at AT			
Baseline	29.9 ± 4.9*	31.0 ± 5.4	0.705
Post-EECP	27.0 ± 4.5*	30.6 ± 5.8	0.234
6-month	28.3 ± 2.6	31.2 ± 6.3	0.317

* $p <$ 0.05 comparing the measurement at post-EECP or 6-month follow-up to that of baseline; † $p < 0.05$ comparing measurement at post-EECP and 6-month follow-up. pVO_2, peak oxygen consumption; RER, respiratory exchange rate; VE/VCO_2, ratio of ventilation to carbon dioxide production.

Table 6.2.6 Bioimpedance estimated body compositions at baseline, post-EECP and 6-month follow-up

	Control n = 6	Active n =8	*p*
Systolic blood pressure (mmHg)			
Baseline	123 ± 23	118 ± 17	0.593
Post-EECP	114 ± 16	111 ± 16	0.747
6-month	126 ± 12	110 ± 13	0.037
Diastolic blood pressure (mmHg)			
Baseline	72 ± 12	68 ± 10	0.561
Post-EECP	65 ± 11	62 ± 13	0.654
6-month	72 ± 13	59 ± 5	0.032
Heart rate (bpm)			
Baseline	64 ± 11*	68 ± 13	0.572
Post-EECP	59 ± 9*	64 ± 6	0.270
6-month	64 ± 10	68 ± 10	0.433
Weight (kg)			
Baseline	91.4 ± 31.2	86.9 ± 18.7	0.754
Post-EECP	90.8 ± 29.1	86.1 ± 16.6	0.708
6-month	91.7 ± 30.1	85.8 ± 16.7	0.694
Fat-free mass (kg)			
Baseline	63.4 ± 19.8	60.1 ± 12.4	0.726
Post-EECP	59.7 ± 13.7	59.2 ± 10.3	0.932
6-month	61.0 ± 14.3	58.8 ± 8.9	0.727
Fat mass (kg)			
Baseline	28.0 ± 13.1	26.8 ± 9.2	0.843
Post-EECP	31.0 ± 17.7	26.9 ± 9.7	0.582
6-month	30.7 ± 17.7	27.0 ± 9.8	0.630
Total body water (kg)			
Baseline	46.4 ± 14.5	44.0 ± 9.1	0.726
Post-EECP	43.7 ± 10.0	43.3 ± 7.5	0.935
6-month	44.7 ± 10.5	43.1 ± 6.6	0.730
Basal metabolic rate (kcal)			
Baseline	1789 ± 489	1687 ± 311	0.658

Post-EECP	1781 ± 463	1673 ± 277	0.595
6-month	1790 ± 476	1668 ± 276	0.555
Impedence (ohms)			
Baseline	475 ± 159	460 ± 100	0.845
Post-EECP	501 ± 114	472 ± 88	0.600
6-month	484 ± 109	480 ± 69	0.930
* $p <$ 0.05 comparing the measurement at baseline and early post-EECP			

Table 6.2.7 Laboratory results for blood and 24-hour urinary tests at baseline, post-EECP and 6-month follow-up

	Control n = 6	Active n =8	*p*
Blood tests			
Haemoglobin (g/dL)			
Baseline	14.6 ± 1.0	13.9 ± 1.7	0.379
Post-EECP	14.6 ± 1.1	13.7 ± 1.3	0.180
6-month	14.8 ± 1.2	14.0 ± 1.4	0.28
White cell count (x 10^9/L)			
Baseline	6.0 ± 0.9	7.3 ± 1.4	0.072
Post-EECP	6.2 ± 0.8	7.2 ± 1.5	0.165
6-month	5.7 ± 0.7	6.6 ± 1.9	0.331
Platelet count (x 10^9/L)			
Baseline	178 ± 14	197 ± 34	0.219
Post-EECP	178 ± 27	203 ± 49	0.281
6-month	177 ± 20	199 ± 42	0.259
Plasma viscosity (mPa/s)			
Baseline	1.64 ± 0.07	1.61 ± 0.07	0.506
Post-EECP	1.63 ± 0.08	1.60 ± 0.03	0.465
6-month	1.62 ± 0.08	1.60 ± 0.05	0.607
Sodium (mmol/L)			
Baseline	140 ± 1	139 ± 2	0.602
Post-EECP	139 ± 2	140 ± 2	0.744
6-month	138 ± 2	139 ± 2	0.417
Urea (mmol/L)			
Baseline	5.2 ± 0.9	6.6 ± 3.1	0.330
Post-EECP	5.1 ± 1.8	6.5 ± 2.5	0.278
6-month	4.8 ± 0.9	6.7 ± 3.5	0.223
Creatinine (μmol/L)			
Baseline	100 ± 10	107 ± 28*	0.576
Post-EECP	100 ± 13	101 ± 28*	0.971
6-month	98 ± 6	102 ± 32	0.740
Albumin (g/L)			

Baseline	39 ± 1	41 ± 2	0.095
Post-EECP	40 ± 2	40 ± 3	0.853
6-month	40 ± 1	40 ± 2	0.609
hs-CRP (mg/L)			
Baseline	2.7 ± 2.0	4.0 ± 3.6	0.474
Post-EECP	2.2 ± 2.4	4.5 ± 6.1	0.395
6-month	1.6 ± 1.4	3.6 ± 3.7	0.230
NT-proBNP (pmol/L)			
Baseline	79.0 ± 94.5	78.4 ± 53.3	0.988
Post-EECP	72.3 ± 72.4	85.0 ± 63.0	0.733
6-month	62.5 ± 66.1	85.9 ± 63.5	0.516
hs-Troponin T (ng/L)			
Baseline	9.0 ± 3.4	11.1 ± 9.1	0.607
Post-EECP	9.2 ± 3.5	9.8 ± 7.6	0.876
6-month	10.8 ± 4.6	11.1 ± 8.9	0.937
H-FABP (ng/ml)			
Baseline	4.21 ± 2.18	6.85 ± 5.73*	0.308
Post-EECP	4.70 ± 3.51	5.14 ± 4.23*	0.841
6-month	3.57 ± 1.60	6.55 ± 7.00	0.331
von Willibrand factor activity (%)			
Baseline	61.2 ± 14.7	83.8 ± 26.2	0.083
Post-EECP	65.7 ± 18.8	84.4 ± 31.7	0.225
6-month	64.8 ± 10.3	84.2 ± 37.0	0.240
D-dimer (ng/ml)			
Baseline	85.3 ± 58.5	265.2 ± 347.1	0.237
Post-EECP	91.1 ± 58.8	264.7 ± 407.7	0.326
6-month	84.1 ± 66.9	408.2 ± 257.3	0.276
Fibrinogen (μg/ml)			
Baseline	4920 ± 513*	4957 ± 509	0.896
Post-EECP	4400 ± 497*	4861 ± 1126	0.371
6-month	5106 ± 774	5337 ± 739	0.581
24-hour urinary tests			
Urine volume (mls)			
Baseline	1712.5 ± 469.3	1673.8 ± 677.5	0.907

Post-EECP	1910.0 ± 531.6	1881.9 ± 470.5	0.918
6-month	1598.0 ± 563.7	2005.6 ± 548.1	0.233
Sodium (mmol/24 hrs)			
Baseline	155 ± 81	152 ± 116	0.970
Post-EECP	158 ± 65	166 ± 84	0.841
6-month	131 ± 56	147 ± 61	0.624
Creatinine (mmol/24 hrs)			
Baseline	14.9 ± 5.9	12.6 ± 5.9	0.482
Post-EECP	13.4 ± 3.9	12.3 ± 4.0	0.598
6-month	15.2 ± 3.6	11.0 ± 3.6	0.065
Creatinine clearance (ml/min)			
Baseline	105.8 ± 48.7	83.4 ± 38.5	0.353
Post-EECP	94.0 ± 36.5	84.5 ± 30.9	0.607
6-month	115.0 ± 35.5	72.4 ± 20.4	**0.018**
Albumin (mg/L)			
Baseline	4.6 ± 4.8	6.0 ± 2.3	0.538
Post-EECP	3.8 ± 2.0	7.7 ± 11.7	0.442
6-month	5.6 ± 6.3	3.3 ± 2.9	0.409
Creatinine (mmol/L)			
Baseline	9.0 ± 5.0	8.3 ± 3.4*	0.794
Post-EECP	7.9 ± 4.2	6.3 ± 1.3	0.360
6-month	10.7 ± 2.8	5.6 ± 1.2*	**0.001**
Albumin/Creatinine (mg/mmol)			
Baseline	0.56 ± 0.40	0.77 ± 0.25	0.325
Post-EECP	0.46 ± 0.30	1.11 ± 1.39	0.408
6-month	0.86 ± 0.93	0.63 ± 0.47	0.581

* $p <$ 0.05 comparing the measurement at baseline and early post-EECP.

Normal range: Urinary sodium 140 – 260 mmol/24hrs, creatinine 9.0 – 17.0 mmol/24hrs, creatinine clearance 70 – 140 ml/min, albumin < 30 mg/L and albumin/creatinine ratio <2.5 mg/mmol in men and < 3.5 mg/mmol in women.

H-FABP, heart-type fatty acid-binding protein

Table 6.2.8 Angina control, Duke's Activity Status Index (DASI) and Minnesota Living with Heart Failure Questionnaires (MLHFQ) at baseline, post_EECP and 6-month follow-up

	Control n = 6	Active n =8	*p*
Angina episodes/week			
Baseline	0.8 ± 1.2	5.0 ± 6.0*	0.121
Post-EECP	0.5 ± 0.8	1.3 ± 2.4*	0.487
6-month	0.7 ± 1.2	2.5 ± 4.8	0.381
GTN use/week			
Baseline	1.2 ± 1.5	3.8 ± 6.3	0.339
Post-EECP	0.8 ± 1.3	1.3 ± 2.4	0.713
6-month	0.6 ± 1.2	2.5 ± 4.8	0.360
DASI			
Baseline	22.5 ± 4.1	23.9 ± 5.2	0.605
Post-EECP	22.3 ± 4.4	24.1 ± 5.7	0.535
6-month	22.7 ± 5.2	25.1 ± 5.2	0.397
MLHFQ – total score			
Baseline	37.5 ± 25.8*	39.6 ± 24.5	0.878
Post-EECP	25.0 ± 14.0	35.3 ± 26.3	0.407
6-month	21.0 ± 19.4*	30.0 ± 24.2	0.237
MLHFQ – physical component			
Baseline	17.2 ± 14.6*	18.6 ± 13.0	0.847
Post-EECP	11.3 ± 10.8†	15.3 ± 12.9	0.560
6-month	9.2 ± 9.6*†	15.8 ± 12.7	0.310
MLHFQ – emotional component			
Baseline	7.5 ± 6.7*	7.5 ± 5.4	1.000
Post-EECP	4.0 ± 2.4	8.5 ± 7.2	0.169
6-month	4.7 ± 5.3*	6.8 ± 6.1	0.515

* $p < 0.05$ comparing the measurement at post-EECP or 6-month follow-up to that of baseline; † $p < 0.05$ comparing measurement at post-EECP and 6-month follow-up.

DASI, Duke's Activity Status Index; MLHFQ, Minnesota Living with Heart Failure Questionnaires.

6.2.4 Discussion

In patients with ischaemic heart disease and LVSD, this study showed that conventional EECP treatment regimen improved regional but not global left ventricular wall function and perfusion. There was a sustained improvement in the exercise capacity of the patients receiving conventional EECP treatment regimen but without any associated objective improvement in the other measurements of cardiopulmonary exercise test. With conventional EECP treatment regimen, the objective improvement in regional LV perfusion and function and exercise capacity was associated with a subjective improvement in angina control but not NYHA, DASI or MLHFQ. There was also an associated non-sustained short-term reduction in the H-FABP level following conventional EECP treatment regimen but without any changes in the NT-proBNP and hs-TnT levels.

6.2.4.1 Left ventricular function and perfusion

We have previously shown that dysfunctional myocardial segments is prevalent in patients with IHD and LVSD.[472] Based on LGE CMR study on a group of patients with IHD and LVEF < 50%, more than half of their myocardial segments were dysfunctional. Of these segments, at least a third of them were without any scar and another third had scar in < 50% of the wall thickness.[472] Similar distribution of viable but dysfunctional myocardial segments were observed in both the Controls and Active patients in this study. The cause of myocardial dysfunction in patients with IHD is complex and not limited to the loss of cardiomyocytes following myocardial infarction. It is now clear that full- and partial-thickness infarction with subsequent acute or chronic ventricular remodeling, silent or overt ischaemia with acute and chronic repetitive myocardial stunning, and myocardial hibernation play a role. These pathomechanisms usually co-exist in the same patient and contribute to the progression and manifestation of LVSD and CHF.[478,479] One common pathophysiological factor is the reduction in coronary perfusion reserve which is the main feature of coronary artery disease.[480]

This is the first study to demonstrate the improvement in regional function and perfusion following a typical course of EECP treatment using LGE and gadolinium first pass cardiac magnetic resonance imaging. The improvement in segmental FPRI was apparent following EECP treatment and continued to improve over the 6 months follow-up period. However, the reduction in the number of viable but dysfunctional or

viable but ischaemic segments was only apparent 6 months following EECP treatment. In contrast, the segmental FRPI in the Controls did not changed but deteriorated after 6 months with an associated increase in the number of dysfunctional segment. This suggests that active EECP treatment may improve segmental or regional myocardial perfusion with associated improvement in myocardial function, and that the improvement in myocardial function require a period of time following the improvement in perfusion, and possibly, a certain threshold of improvement in myocardial perfusion is required for functional recovery.

The mechanism(s) of improved perfusion following EECP is unclear. One theory is that EECP potentiates the recruitment of collaterals and promotes angiogenesis.[308] Experimental canine model has shown that EECP increase myocardial capillary density in experimental acute myocardial infarction with associated improvement in myocardial perfusion on radionuclide scan.[309,310] This is related to increased arterial wall shear stress that activates endothelial NO synthase/NO pathway[311] and down-regulates pro-inflammatory cytokines.[312] These inhibit hypercholesterolaemia-induced intimal hyperplasia and development of atherosclerosis by reducing endothelial damage, stopping vascular smooth cell proliferation and migration, and suppressing extracelluer matrix formation.[311] EECP also increases the expression of granulocyte colony-stimulating factor (G-CSF), mobilises endothelial progenitor cells and increases in regional myocardial angiogenesis.[313] In clinical studies, shear stress on vascular endothelium induced by EECP up-regulates the expression of various angiogenic growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatic growth factor (HGF).[314] The presence of collaterals may help protect the myocardium and limit myocardial ischaemia,[481] and hence preventing myocardial dysfunction.

An appropriate level of shear stress is a main factor for maintenance of a functional endothelium.[482] It is known that peripheral endothelial function correlates closely to coronary endothelial function.[321] Coronary endothelial dysfunction is associated with myocardial perfusion abnormality and may contribute to the development of ischaemia, contractile abnormality or progression of left ventricular dysfunction. Indeed, variable degree of coronary endothelial dysfunction present across the spectrum of left ventricular dysfunction.[483] EECP improves endothelial function and modulate endothelial nitric oxide (NO) and endothelin-1 (ET-1) release. EECP causes

dose-dependent increase in NO and decrease ET-1 which can be maintained for up to 3 months after treatment.[315,316] In addition, EECP improves peripheral macro- and/or microvascular endothelial function in patients with symptomatic CAD or patients with LVSD due to CAD.[317-320] The improvement in endothelial function has been shown to be associated with an improvement in the doppler assessment of coronary diastolic peak flow velocity and coronary flow reserve in patients known to have coronary slow flow.[322] This improvement was found to have an inverse relationship with the change in hs-CRP level, suggestive that the effect may be, in part, achieved through modulation of inflammatory pathway or cytokines.[319,322] However, the hs-CRP level did not change in both the Controls and Active patients in this study.

Clinically, EECP improves myocardial perfusion on radionuclide and PET imaging.[304,323-327] In a multicentre observational study that enrolled 175 patients, Stys et al. showed that EECP treatment improved the perfusion defects seen on exercise-treadmill stress radionuclide scan in 54 – 85% of the patients.[324] In 12 patients with stable CAD, Urano et al. has shown that a course of EECP treatment was associated with an improved exercise tolerance and a reduction in the prevalence of exercise-induced reversible perfusion abnormality on thallium radionuclide imaging.[304] Using ^{13}N-ammonia PET scan, Masuda et al. showed that EECP improved myocardial perfusion and coronary flow reserve at rest and with dipyridamole.[323] In addition, EECP also reduces wall motion abnormality during dobutamine-stress echocardiography[328] and this may be related to the severity of coronary disease or the presence of collaterals.[329] However, smaller multi-centre study using technetium Tc 99m sestamibi radionuclide scan[330] and single-centre study using ^{13}N-ammonia PET scan[331] did not show any improvement in myocardial perfusion despite an increase in exercise capacity following EECP treatment. It is plausible that although the majority of patients benefit from EECP via improved myocardial perfusion, certain subgroups of patients less likely to benefit from the treatment. IEPR data has shown that patients with heart failure and diabetes are less likely to gain angina relief following EECP.[344,345] In contrast, patients with a prior CABG may be more likely to experience angina relief following EECP,[484] whilst the number of significantly diseased coronary artery can adversely affect the improvement in radionuclide stress perfusion imaging.[327]

The improvement in the function of viable myocardial segments following active EECP treatment may be, at least in part, related to the improvement in

myocardial perfusion. In patients with dysfunctional myocardial segments in the presence of underlying significant obstructive coronary lesions, coronary revascularisation especially by mean of conventional coronary artery bypass surgery (CABG) may lead to improvement in the contractile function of viable myocardium.[475,476] Kim et al has shown in 41 patients with stable coronary disease that 10 weeks following CABG, dysfunctional myocardial segments with < 25% wall thickness of LGE had a high likelihood of functional recovery; whilst dysfunctional segments with > 50% wall thickness of LGE had little potential to recover.[485] This spectrum in the potential for functional recovery is due to the fact that hibernating myocardium has a continuum of histological and biochemical perturbations that define its ability to recover.[486,487]

In addition, myocardial functional recovery following EECP may be partly attributed to factors other than the improvement in myocardial perfusion. EECP has been shown to improve oxygen metabolism in ischaemic myocardium.[458] EECP treatment is also associated with a dose-dependent reduction in plasma markers of oxidative stress.[293,488] This suggest a potential benefit of EECP treatment in LVSD and CAD as oxidative stress has been implicated as an important factor in cardiovascular disease progression and manifestation including atherosclerosis, vascular and myocardial remodeling, endothelial dysfunction as well as myocardial stunning and hibernation.

Raised LV end-diastolic pressure (LVEDP) is known to increase myocardial oxygen demand[489] and decrease the perfusion pressure for coronary filling[490] leading to myocardial ischaemia in the presence of CAD. EECP can improve LV diastolic filling and reduce LVEDP[304] which may in turn, reduce myocardial ischaemia. In post-infract patients, a higher LVEDP can lead to unfavourable myocardial remodelling and LV dilatation.[491] The reduction in LVEDP following EECP treatment may reduce LV wall stress and attenuate or reverse unfavourable remodelling in patients with LVSD.[492] This may explain, although statistically insignificant, the reduction in LVEDV and LVESV observed in the Active patients in this study. EECP is also associated with reduction in the levels of BNP or NT-proBNP and atrial natriuretic peptide (ANP) in patients with CAD. The reduction was observed after one treatment session and continued to decrease one week after a course of treatment. This effect was maintained for up to one month after the treatment.[323,493] The reduction in BNP is directly related to the reduction in

LVEDP.[304] However, the level of NT-proBNP in the Controls and Active patients did not change in this study. The reason is unclear but a plausible explanation is that the patients included in this study had stable heart failure with a relatively low baseline NT-proBNP level.

EECP also favourably modulates the renin-angiotensin system (RAS). RAS plays an important pathophysiological role in LVSD and CAD and has been the strategic target for heart failure treatment.[332] A course of EECP is associated with significant reduction in plasma renin, angiotensin converting enzyme and angiotensin II levels.[333] This suggests a potential benefit of EECP in heart failure patients.

However, EECP can also improve left ventricular function independent of changes in haemodynamics. A course of EECP was found to be associated with significance increase in left ventricular preload-adjust maximal power and ejection fraction.[306] Similarly, using bioimpedence measurement, EECP increases maximum cardiac power by 32% in patients with LVSD and CAD.[307]

In contrast, some evidence has suggested EECP may also exert a peripheral effect similar to that of exercise training.[325,334] This is not surprising as EECP may theoretically cause passive mechanical stimulation of lower limb muscles and brings about various beneficial effects to heart failure patients similar to the effects of exercise training.[335,336] Exercise training may improve diastolic and LVEF in patients with CHF due to LVSD.[494]

However, segmental wall motion and perfusion improvement following EECP therapy did not translate into global LV function or perfusion improvement in this study. Indeed, the cardiac output (CO) in the Active patients decreased following EECP treatment before returning to baseline level after 6 months follow-up. Although similar trend was observed in the stroke volume (SV) or LVEF of these patients, the results were statistically insignificant. These findings may be related to an early reduction in the LVEDV following EECP followed by a delayed reduction in LVESV after 6 months suggestive of favourable LV remodelling. However, this observation was not statistically significant, possibly related to the small number of patients recruited into the study. In contrast, the Controls had significant reduction in the LVEF due to an increase, although statistically insignificant, in the LVESV that may represent unfavourable LV remodelling. Study using thoracic electrical impedance in patients

with chronic stable angina by Arora et al. may help to explain some of this findings.[300] A course of EECP was found to reduce stroke volume and index of LV contractility with little change in thoracic fluid content. There was also a reduction, but statistically insignificant, in LV end-diastolic index that represents preload. In combination with earlier discussion, it is possible that a course of EECP may improve LV diastolic filling and pressure leading to lower LV wall tension, myocardial contractility and work load with favourable reverse remodelling and lower SV but stable LVEF.

The effect of EECP on global LV function has not been well investigated in patients with CHF and LVSD. The prospective Evaluation of Enhanced External Counterpulsation in Congestive Heart Failure Study (PEECH) randomised 187 patients with LVEF <= 35% to optimal medical therapy versus optimal medical therapy and EECP showed a benefit, especially in patients older than 65 years of age, in exercise tolerance, symptom and quality of life but the effect on LV function was not reported.[292,297,349] In an observational study of 47 patients with CHF due to CAD, the LVEF improved from 32 ± 17 to 36 ± 15% after a course of EECP.[305] Much of the other experience on EECP and LV function has involved patients with angina and preserved systolic function. Based on echocardiographic examination including the use of tissue Doppler study, Esmaeilzadeh et al. has shown that a course of EECP increased both systolic and diastolic function of 20 patients with symptomatic coronary artery disease and their LVEF improved from 40 ± 13% to 46 ± 13%.[495] An associated reduction in BNP but not hs-CRP level was observed in the same study. In 14 patients with CAD, dobutamine stress echocardiographic study has shown that EECP increased resting LVEF from 47.2% to 52.1% and peak stress LVEF from 65.3% to 70.3% early after a course of treatment without any effect on the LV diastolic function.[496] In contrast, Kumar et al. did not find any change in the echocardiographic LV systolic and diastolic function of 47 patients with CAD and mean LVEF of 42 ± 8% following a course of EECP.[497]

The reason for absence of global LV functional improvement in this study is unclear but may be explained by the findings from randomised control studies investigating the role of conventional revascularisation in patients with LVSD due to significant underlying obstructive coronary disease. The Heart Failure Revascularisation Trial (HEART) randomised 138 patients with coronary artery disease and LVEF < 35% with at least 5 segments, in a 17-segment model, of viable myocardium to optimal

medical therapy versus conventional percutaenous or surgical revascularisation in addition optimal medical therapy. After a median follow-up of 59 (interquartile 33 – 63) months, the mortality was 37% in both groups of patients without any improvement in LV systolic function.[432] The Surgical Treatment for Ischemic Heart Failure Trial (STICH) randomised 1212 CHF patients with LVEF < 35% to optimal medical therapy alone versus medical therapy and CABG showed that CABG did not confer survival benefit even in a subgroup of patients with viable myocardium.[433,498] Recently, it becomes apparent that a substantial extent of viable myocardium (>= 10 segments in a 16 segment-model) is required for an absolute 3% improvement in the LVEF following CABG based on a CMR study.[499]

6.2.4.2 Cardiopulmonary exercise test

We observed that the exercise time improved significantly in the Controls early after their treatment but the increase was not sustained by 6 months. In contrast, there was an increase in the exercise time of the Active patients immediately following EECP and this continued to increase over the 6-month follow-up period. However, there was no associated increase in the ventilation and oxygen consumption components of the CPEx test including the pVO_2 and anaerobic threshold. These findings are similar to those of the PEECH study.[292]

The absence of an increase in the pVO_2 and anaerobic threshold, better measurements of exercise capacity, raised the possibility of a placebo effect from EECP treatment. However, the continuing increase in exercise time in the Active patients as opposed to the transient exercise time increase in the Controls would suggest a true beneficial treatment effect from the conventional EECP treatment regimen. The pattern of increase in exercise time in the Active patients is also similar to that of the improvement in segmental FPRI. Further, the pre-specified subgroup analysis in PEECH study showed an improvement in the exercise time and pVO_2 in patients who were older than 65-years of age.[297] A small increase in pVO_2 occurs during a session of EECP can be observed.[334] This increase is equivalent to a very low level of exertion and unlikely to induce a significant training effect. However, the minimal effective exercise intensity for increasing cardiorespiratory fitness in unfit or fit patients with and without CAD is lower than previously observed, 30% - 45% of the VO_2 reserve.[337] It remains plausible that EECP may have a sufficient peripheral training effect among unfit

patients with significant cardiovascular disease such as heart failure or refractory angina.[325,334]

6.2.4.3 Laboratory tests

The Active patients had a small and short-term reduction in the serum creatinine level following EECP treatment. This was accompanied by insignificant reduction in the urinary creatinine and creatinine clearance. Although this may be related to small number of patients recruited, there may be a valid pathophysiological explanation. In 30 patients referred for EECP treatment predominantly for angina [7/30 for CHF, median LVEF 55 (IQR 18)%], the serum c-cystatin level reduced with an associated improvement in the GFR especially in those with GFR < 60 mls/min/1.73m^2 or NT-proBNP > 125 pg/ml immediately after EECP.[356] This improvement was sustained for up to 16 months. Therefore, the short-term reduction in creatinine may be partly related to a temporary increase in creatinine clearance which may in turn, a result of an increase in renal blood flow and perfusion following EECP treatment.[301] Since creatinine is a breakdown product of creatine phosphate in the muscle and EECP may have low level exercise training effect, the lower level of serum creatinine may be related to a reduction in muscle wasting which occurs in heart failure and may be preventable by exercise.[500] However, there was no change in the fat free mass and total body water in the patients based on the bioimpedance measurements. Another non-randomised study of 47 patients with CHF and CAD did not show any change in creatinine level immediately after EECP.[305]

With the improvement in segmental wall motion abnormality in the Active patients, one would envisage that EECP treatment can reduce the level of natriuretic peptides. However, as mentioned earlier, the NT-proBNP levels in both Active patients and Controls in this study did not change and may be related to the fact that patients recruited into this study had stable CHF with low baseline NT-proBNP level which was unlikely to improve further. In contrast, the only other study involved heart failure patients (n=47) and had BNP measurement showed a reduction in BNP level early following active EECP treatment.[305] Laboratory results were not reported in the PEECH study. In patients treated with EECP for angina, the ANP and BNP or NT-proBNP levels are lower immediately after a course of treatment and continue to decrease in the following week. The reduction is sustained for at least a month[323,493] and is directly related to the reduction in LVEDP.[304]

An interesting finding in this study was a short-term reduction in the level of H-FABP in the Active patients early after EECP treatment. In patients with CHF, on-going myocardial damage may be one of the pathophysiological processes responsible for unfavourable remodelling and is detectable by measurement of serum cardiac troponins[400] or H-FABP.[23] However, the level of hs-troponin T in both the Controls and Active patients did not change. As discussed earlier, H-FABP is a cytosolic protein and may be a more sensitive and dynamic markers of on-going myocardial damage.[22] The reduction is H-FABP early after EECP may support that active EECP therapy may lead to some degree of reverse remodelling given the improvement in regional wall motion abnormality and the reduction, though statistically insignificant, LVEDV and LVESV seen in this study. However, as discussed earlier, H-FABP level can be affected by renal function and the reduction in H-FABP seen in this study may also be directly related to the reduction in creatinine.

EECP has been shown to favourably affect inflammatory markers via increasing endothelial shear stress in patients with CAD and may therefore suppress the low-grade inflammation associated with cardiovascular condition. In hypercholesterolaemic porcine model, EECP improves vascular flow mediated dilatation and reduces hs-CRP level.[501] Casey et al. randomised 21pateints with CAD to conventional EECP (cuff inflation pressure 300mmHg) versus sham placebo (cuff inflation pressure 75mmHg) and found reductions in alpha tumour necrosis factor and mococyte chemoattractant protein-1 in patients who had a course of conventional EECP treatment.[502] EECP also reduces inflammatory markers in patients with impaired glucose tolerance.[503] Lou et al also found that in 45 patients with coronary slow flow, a course of EECP reduced hs-CRP level with an associated improvement in forearm flow mediated dilation and doppler assessment of coronary diastolic peak flow velocity and coronary flow reserve.[322] However, the hs-CRP levels of the Controls and Active patients in this study did not change following EECP treatment. This is consistent with Kozdag's study on patients with CHF due to CAD.[305] Perhaps the degree of inflammatory response is more profound once heart failure sets in.[65]

Patients with CHF are thought to have hypercoagulable state due to a combination of factors including endothelial dysfunction that constitute Vichow's triad.[32] There is theorectical possibility that EECP may have an impact on abnormal haemostasis in patients with CHF by improving vascular endothelial function. However,

EECP does not alter the level of D-dimer, vWF, tissue plasminogen activator and plasminogen activator inhibitor-1 in patients with CAD.[504] This is consistent with the findings of this study as the level of D-dimer, fibrinogen and vWF did not change in the Active patients. The reason for a short-term reduction in fibrinogen seen in the Controls is unclear. It may be related to an increase in clot formation since there was a corresponding non-significant and small increase in the D-dimer level, or a reduction in hepatic fibrinogen synthesis.[505]

6.2.4.4 Symptoms and Quality of life

Unexpectedly, the Active patients experienced an improvement in their angina frequency following EECP treatment but without any improvement in NYHA, DASI or MLHFQ. The improvement in angina frequency was not sustained at 6-month follow-up. In 71 of 139 patients with stable angina randomised in the Multicentre Study of Enhanced External Counterpulsation (MUST-EECP), Health-related Quality of Life (HQOL) data were collected.[291,340] After 1 year, sustained improvement in all aspects of HQOL including reduction in bodily pain, increased social functioning, improvement in physical function and quality of life was seen in the patients who received active EECP treatment.[340] From a subgroup of the patients enrolled in The International EECP Patient Registry Phase 2 (IEPR-2) for treatment of refractory angina, angina control and DASI improved by similar extent in 111 patients with LVEF <= 35% and 366 patients with LVEF > 35%.[506] In another group of 139 patients with CHF and refractory angina (mean LVEF 35 ± 16%) enrolled in the IEPR-2, we have shown that EECP improved angina control and NYHA with associated improvement in DASI as well as all components of Kansas City Cardiomyopathy Questionnaires (KCCQ).[425] The improvement in KCCQ was seen in both the clinical and functional entities of the questionnaire. Moreover, PEECH study[292] and an earlier Multicentre Feasibility Study of EECP in CHF patients[348], sustained improvement in MLHFQ over 3 to 6 months was reported.

On the other hand, the improvement in MLHFQ in the Controls without a sustained improvement in exercise tolerance and other objective measurements is highly suggestive of an element of placebo effect.[339]

6.2.4.5 Adverse effects

Two patients had adverse event and withdrew from taking part in the study, one had TIA with transient facial hemiparaesthesia and the other had aggravation of lower lumbar back pain. As mentioned earlier, these patients did not agree to return for follow-up and hence were excluded from this analysis. Other patients did not experience any treatment adverse event nor had any cardiovascular event during the 6-months follow-up period.

6.2.4.6 Comparison to PEECH

The design of this study was aimed to extend findings beyond those of PEECH.[349] By having non-treatment group of patients as controls, the results from PEECH may not mitigate against the possibility that daily contact with healthcare personnel in the active treatment group might have benefited the patients. Although this study has taken into account the effect of regular healthcare contact, it was unethical to withhold information from patients that the conventional treatment regimen constituted 35 1-hour sessions. However, effort was made to prevent contact of all patients during treatment and followed period. As we previously discussed, it is difficult to identify a satisfactory control group for therapy such as EECP in order to take into account of the potential placebo effect.[338] Low pressure sham placebo is not without haemodynamic effect[302] and may have a degree of peripheral training effect.[507] There is theoretical concern that increase venous return with inadequate systolic unloading may lead to decompensated heart failure. In an elegant invasive haemodynamic study, Taguchi et al. has shown that the right atrial pressure (RAP) and pulmonary capillary wedge pressure (PCWP) increased by 15 minutes of EECP treatment with concomitant reduction in the systemic vascular resistance.[261] Both RAP and PCWP only returned to baseline after 45 minutes of treatment. Therefore, the 5-minute regimen was thought to be a safe and suitable control.

Further, there has not been any study specifically investigates the effect of EECP has on symptoms/quality of life, functional status and myocardial perfusion and function of patients with CHF. To date, only two other observational studies specifically aimed at patients with CHF are available.[305,348] This study is the first study to investigate the effect of EECP has on myocardial perfusion and function in patients with CHF. It is also the first study to use CMR in the assessment of LV function and perfusion.

6.2.4.7 Limitations

The main and obvious limitation of this study is the number of patients recruited. The main reason was that soon after the commence of the study, the result of Cardiac Resynchronisation – Heart Failure study (CARE-HF) was reported.[369] As patients suitable for this study were very often candidates for cardiac resynchronisation therapy (CRT), it was thought to be unethical to delayed their CRT implantation in order to accommodate them having CMR within the protocol of this study. CMR compatible device and CRT leads were not widely available and there were few reports regarding the safety of CMR in patients with CRT, hence it was thought that CMR should only be performed in these patients for a valid clinical indication rather than research purposes. Although we had experience in three-dimensional echocardiography for LV assessment[508], it was not readily available to us for the expected time frame of the study.

Nevertheless, the number of CHF patients required for study using CMR is generally small.[509] For example, 8 patients will be adequate to detect a 10mls change in the SV with a 90% statistical power and an α error of 5% as compared to 69 patients required if two-dimensional TTE was used. Similarly, 14 CHF patients are required for detecting a 3% absolute change in LVEF using CMR instead of 115 patients required if using two-dimensional TTE. Therefore, the findings in this study, though not conclusive, may serve as hypothesis generating.

Although patients recruited into this study were on optimal and stable CHF medications, none of them had CRT that confers symptomatic and survival benefit to the majority of a selected group of patients.[369] Therefore any potential benefit of EECP has to be examined as an adjuvant therapy in addition to optimal medications and CRT or optimal medications only in patients who are not suitable to receive a CRT.

6.2.5 Conclusion

Enhanced external counterpulsation may improve regional myocardial perfusion and function in patients with stable CHF due to LVSD and on optimal medications. There may be associated favourable reverse remodelling but the overall effect it has on global LV function remains unclear. EECP can also improve angina control and exercise tolerance in these patients but the element of placebo effect cannot be fully

excluded. The inadequate sample size in this study may also preclude a conclusion to be made on how EECP affect the quality of life in patients with CHF and LVSD.

Chapter 7

Conclusion, acknowledgements, publications, appendix and references

7.1 Conclusion

The cross-sectional study on biomarkers showed that the prognosis of patients with CHF due to LVSD remains poor despite modern heart failure treatment. In a cohort of close to 500 patients on stable treatment with almost 90% prescription rate for an ACEI or ARB and β-blocker, the mortality rate was approximately 50% in a mean follow-up period of 78.1 ± 6.1 (range 67.5 – 89.6) months. The 5-year mortality rate was 40.4% and approximately ¾ of the deaths were due to a cardiovascular cause. Nonetheless, this is a relatively significant improvement in the prognosis compared to the historical figure of 50% 5-year mortality rate with an annual mortality of approximately 10%.[413,510] Although an improvement, this survival rate is still worse than many other illnesses such as cancers. Therefore, still much is needed to be done to improve the prognosis of patients with CHF, but improvement in the quality of life should not be neglected.

During the course of the recruitment and follow-up period of the patients within the studies of this thesis, a few landmark randomised control trials (RCTs) have reported treatment that may have significant impact on the prognosis of patients with CHF or clarified some controversies in the treatment of these patients. The CArdiac REsynchronisation Heart Failure (CARE-HF) Study confirmed a significant prognostic benefit of cardiac resynchronisation therapy (CRT) in patients with CHF and established such device treatment as the standard modern heart failure treatment regimen in suitable patients.[1,369] Although not directly related to the topics of this thesis, the change in practice affected the enrolment of patients into the study of Enhanced External Counterpulsation (EECP) in CHF patients due to the incompatibility of CRT devices and Cardiac Magnetic Resonance Imaging (CMR) that was the primary investigational tool of the study design.

It haslong been established that patients with CHF have perturbed rheology and haemostasis leading to a hypercoagulable state.[32,55] This is thought to be due to various mechanisms including neurohormonal and inflammatory activation, leading to abnormal blood constituents and flow as well as abnormal vessel wall due to endothelial

dysfunction – combination of factors traditionally known as the Vichow's triad.[32] As discussed in the introduction, clinical and post-mortem studies have confirmed a high prevalence of thromboembolic disease in patients with CHF and perhaps, a large number of such events are not clinically apparent.[47]

The cross-sectional study of haemostatic markers in this thesis involved one of the largest cohort of patients with stable CHF and confirmed that these patients have deranged haemostasis when compared to patients without CHF.[371] Importantly, it is the first large scale study of haemostatic markers in patients with stable CHF due to LVSD which showed that D-dimer and vWF activity were independent prognostic markers after taking into account a few conventional prognostic markers including NT-proBNP.

However, RCTs have not convincingly shown that anti-thrombotic therapy alters the course or prognosis of heart failure in patients with sinus rhythm. Combined data analysis from Wafarin/Asprin Study in Heart failure (WASH) and Warfarin and Antiplatete Therapy in Heart failure Trial (WATCH) studies suggests that aspirin may increase the risk of hospitalisation due to decompensated heart failure when compared to warfarin in these patients.[45,46,48] The HEart failure Long-term Antithrombotic Study (HELAS) randomised patients with CHF due to ischaemic heart disease (IHD) to aspirin or warfarin and those with dilated cardiomyopathy (DCM) to placebo or warfarin.[49,50] The outcome was neutral with low incidence of thromboembolic events. The recent Warfarin versus Aspirin in patients with Reduced Cardiac Ejection Fraction (WARCEF) study was neutral but post-hoc analysis has shown that younger patients may benefit from warfarin therapy and warfarin may be beneficial in reducing the risk of cardioembolic ischaemic stroke.[51-53] Therefore, patient selection may be the key issue when considering antithrombotic therapy in patients with CHF and in sinus rhythm. Haemostatic markers may help to identify patients who may potentially benefit from anti-thrombotic treatment. We found that D-dimer, a marker of thrombogenesis, was an independent prognostic marker for mortality and cardiovascular hospitalisation and its level was static in stable patients with CHF making it a possible marker that help stratify the risk of patients with CHF.

Another example of the potential use of biomarker to identify patients who may benefit from certain treatment comes from the study of statin use in patients with CHF. Prior to RCTs, many observational data including our community-based study[511] have shown that statin was associated with better prognosis in patients with CHF. We found

that CHF patients who were not on a statin or had their statin treatment withdrawn had a worse outcome than those who were taking or commenced on a statin following enrolment into our local heart failure clinic. However, two RCTs of statin in patients with CHF, the Controlled Rosuvastatin Multinational Study in Heart Failure (CORONA)[380] and the Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico-Heart Failure Trial (GISSI-HF)[381], have subsequently found that statin does not confer better prognosis. Nevertheless, post-hoc analyses in CORONA study have suggested that patients with lower NT-proBNP[382] and higher hs-CRP[383] can benefit from rosuvastatin.

In line with abnormal blood constituents, various studies have identified anaemia, abnormal red blood cell or haematopoiesis with consequent high red cell distribution width (RDW) and higher white cell count or altered distribution of its component carry prognostic information in patients with CHF. However, most of the studies considered these components of routine full blood count (FBC) separately and few have combined both red and white cell variables. As FBC is a routine blood test performed in general clinical setting especially when patients have their first contact with the secondary healthcare setting, we investigated the prognostic value of FBC based on the first FBC of consecutive patients attended our heart failure clinic. We found that RDW in combination with a white cell components, namely neutrophil and lymphocyte or neutrophil-to-lymphocyte ratio, have incremental prognostic value over a few conventionally established prognostic factors including NT-proBNP.

CHF is also associated with activation of inflammatory response that is known to be involved in the progression of heart failure. We have previously shown that the level of hs-CRP was raised in patients with stable CHF and that higher level of hs-CRP was associated with a worse prognosis.[69] Since different subgroups of white cell have specific role in inflammatory process, simple and inexpensive white cell differential test may help to guide specific therapeutic target as oppose to the general reflection of inflammation by hs-CRP. As higher neutrophil and lower lymphocyte counts and higher neutrophil-to-lymphocyte ratio are associated with poorer prognosis, more complex immunomodulation therapy specifically targeted at preventing neutrophil activation or/and lymphocyte apoptosis may be of prognostic benefit in patients with CHF. Standard immunosuppressant therapy in CHF is not beneficial but may lead to harmful outcome as reported in the Anti-TNF Therapy Against Congestive Heart Failure

(ATTACH) trial[71] and Randomised EtaNErcept Worldwide evaluation (RENEWAL) trial.[512]

In addition to abnormal neurohormonal factors, inflammatory response and haemostasis, abnormal myocardial substrate also contributes to the progression of heart failure and affects the patients' prognosis. Even stable patients with CHF and on optimal modern heart failure treatment continue to experience on-going myocardial damage which may contribute to unfavourable myocardial remodeling and progression disease. This concept is based on the detection of cardiac troponins in the serum of clinically stable patients with CHF, suggestive of myofibrillar and myocyte damage. Even low level of detectable cardiac troponins is associated with an adverse outcome in these patients. As H-type fatty acid-binding protein (H-FABP) is a cytosolic protein that can be released rapidly into the blood upon myocardiocyte injury even without necrosis and the level rapidly returns to baseline levels within 20 hours even in the context of myocardial infarction, it is a more dynamic and sensitive marker of myocardial damage than cardiac troponins.[22]

Our cross-sectional study showed that patients with CHF due to LVSD had higher level of H-FABP irrespective of the underlying aetiology of LVSD. Our study represents the largest cohort of unselected patients with CHF as all of the previous studies of H-FABP were small and included only patients with acute decompensated heart failure or excluded patients with moderate and severe renal impairment. Our study showed that H-FABP was higher in patients with LVSD than patients without LVSD or a small group of normal subjects. We found that H-FABP was an independent predictor of mortality and may have added prognostic value over other conventional prognostic markers including NT-proBNP.[397] We also showed that some patients had persistently high H-FABP even after initiation and optimization of their heart failure treatment and these patients had a worse outcome than the others. Further longitudinal study on a subgroup of patients with stable CHF showed that some of these patients had persistently raised H-FABP and an increase in H-FABP level despite having stable clinical status and these were the patients who had a worse long-term outcome. Therefore, H-FABP can potentially be a useful marker to help monitoring treatment response and stratifying the risk of patients with CHF due to LVSD. Further prospective study, in conjunction with the measurement of cardiac troponins, may help validate the findings in these studies.

Myocardial substrate and perfusion are also areas of interest in research to stratify risk and identify potential beneficial treatment in patients with LVSD, especially those due to underlying CAD. CMR and late gadolinium enhancement imaging (LGE) has improved the accuracy and understanding in assessing the left ventricular function and myocardial substrates of patients with CHF. We have shown that in patients with LVSD due to CAD have high prevalence of viable but dysfunctional myocardial segments based on a 17-segment LV model.[472] Observational studies, mainly in patients underwent coronary artery bypass graft surgery (CABG), have shown that myocardial segments with scar thickness less than 50% of the myocardial wall thickness have the potential to recover in contractile function following revascularization.[476]

However, RCTs of revascularisation in patients with CHF due to CAD failed to convincingly establish a definitive beneficial effect when compared to optimal medical therapy alone. The HEArt failure Revascularisation Trial (HEART) randomised 138 patients with LVEF < 35% with at least 5 viable myocardial segments, in a 17-segment model, to conventional surgical or percutaneous revascularisation compared to optimal medical therapy alone did not show any prognostic benefit in patients who underwent revascularisation in addition to optimal medical therapy.[432] However, the study was under-powered due to early termination by funding withdrawal secondary to slow recruitment. The Surgical Treatment for Ischemic Heart Failure (STICH) trial did not show any survival benefit from revascularisation with CABG in addition to optimal medical therapy when compared to optimal medical therapy alone in patients with LVSD due to CAD.[433] However, there may be potential benefit if cardiovascular hospitalisation was taken into account. Nevertheless, in a subgroup of patients with viability assessment by single-photon emission computed tomography (SPECT) or dobutamine-stress echocardiogram, patients with viable myocardium fare better following CABG. However, myocardial viability was not an independent predictor of outcome following CABG.[498] Therefore, a less invasive strategy should be adopted in the management of these patients. It is also apparent from more recent work that a substantial extent of viable myocardium (>= 10 segments in a 16 segment-model) is required for a small improvement in global LV function (an absolute 3% improvement in the LVEF) following CABG based on a CMR study.[499]

EECP improves diastolic perfusion pressure (diastolic augmentation) and reduces myocardial workload and oxygen requirement during systole (systolic unloading). Following a course of treatment, the beneficial effects could be sustained

for up to 3 years, but a smaller study has shown benefit up to 5 years. Most data available are limited to observational studies and the majority have shown an improvement in myocardial perfusion by means of either SPECT, positron-emission tomography (PET) or dobutamine-stress echocardiogram. There may also be beneficial haemodynamic effects or direct effect on the myocardium leading an improvement in LV function, load-dependent or load-independent. The mechanism(s) is unclear but multiple factors have been proposed including collateral recruitment and angiogenesis, neurohormonal, improvement in endothelial function, direct myocardial effect or peripheral training effect. However, an element of placebo effect has not been excluded. Historically, EECP was trialed in patients with acute myocardial infarction or cardiogenic shock. The randomised sham placebo control Multicenter Study of Enhanced External Counterpulsation (MUST-EECP) then showed that EECP was safe and effective in the treatment of stable angina and improved exercise tolerance with sustained benefit in angina control and quality of life for up to 1 year.[291,340] It was later thought to be potentially beneficial in patients with CHF.

As one of the first centres in the UK and Europe, our previous pilot study showed that EECP was safe and effective in improving angina control and exercise tolerance in patients with refractory angina.[435] As a participating centre for the International EECP Patient Registry (IEPR), we later reported that the beneficial effects of EECP in patients with refractory angina could be sustained for up to 3 years in the majority of patients enrolled in the registry.[438] EECP was also safe and effective in patients with advanced CAD who had a prior laser myocardial revascularisation and experienced a recurrence of their angina.[439] Based on the IEPR data, we have also reported that EECP was safe and effective in improving angina control, Duke Activity Status Index and quality of life using heart failure disease-specific Kansas City Cardiomyopathy Questionnaire.[425]

The Prospective Evaluation of Enhanced External Counterpulsation in Congestive Heart Failure (PEECH) study has shown that EECP improved exercise tolerance, symptom and quality of life in patients with CHF and NYHA II or III functional state when compared to non-treatment controls.[292] Although the benefit was sustained for at least 3 months, objective improvement in peak oxygen uptake was only observed in a pre-defined subgroup of patients old than 65 years of age.[297] The potential placebo benefit from regular contact with healthcare in the treatment group could not be

excluded. Further, the effect on LV function, myocardial perfusion and laboratory tests were not available.

The RCT of EECP in patients with LVSD and CAD in this thesis was planned to extent the findings in PEECH and eliminate the potential placebo effect by having brief treatment regimen for the controls. However, it was not feasible to enroll the required number of patients into the study. Nevertheless, the data available suggest that EECP can improve exercise tolerance with objective improvement in regional myocardial perfusion and function assessed by CMR. The study also found that EECP may have favourable effect on myocardial remodeling and on-going myocardial damage by the reduction in left ventricular volumes and serum H-FABP level. CMR-compatible CRT devices may allow further study of the potential usefulness of EECP in patients with CHF with persistent symptom despite CRT.

There are limitations in the studies within this thesis and they have been discussed in detailed within the relevant chapters. All the observational studies in this thesis were, in principle, pilot studies without power calculation mainly due to a lack of prior studies to provide satisfactory references. Hence, the studies are very much hypothesis generating but, by the nature of including consecutive patients without specific exclusion criteria, the results are representative of what could be expected in real-world clinical setting.

7.2 Publication from this thesis

1. PH Loh, J Windram, A Louis, E Kennard, AS Rigby, J Cook, S Kelsey, N Nikitin, JGF Cleland. The immediate and two-year outcomes of enhanced external counterpulsation (EECP) in the treatment of chronic refractory angina – A United Kingdom (UK) perspective. [Abstract] **Heart 2005;91(Suppl 1):223**
2. PH Loh, A Louis, J Windram, J Cook, AS Rigby, J Bryce, L Ingle, N Nikitin, J Cleland. Enhanced external counterpulsation (EECP) improves angina control and exercise tolerance in patients with chronic stable refractory angina. [Abstract] **Heart 2005;91(Suppl 1):224**
3. PH Loh, J Windram, E Kennard, J Cook, S Nabb, S Kelsey, A Louis, JGF Cleland for the International EECP Patient Registry (IEPR). Impact of enhanced external counterpulsation (EECP) on symptoms, health status and medication use in patients with both angina and heart failure. [Abstract] **Eur J Heart Fail Suppl 2005;4:112-3**
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7.3 References

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7.4 Appendix

7.4.1 Ethics Committee Approvals

HULL AND EAST RIDING LOCAL RESEARCH ETHICS COMMITTEE

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Dr. S Chattopadhyay
Clinical Lecturer in Cardiology
Adacemic Dept of Cardiology
Castle Hill Hospital
Cottingham
HU16 5JQ

30 July 2002

Dear Dr Chattopadhyay,

LREC/ 06/02/117
Prevalence of haemostatic abnormalities in patients with heart failure compared to patients with ischaemic disease without heart failure and healthy volunteers: Part I - its characterisation and changes over time; Part II - its relation to the aetiology of heart failure

The Chair of the Hull and East Riding REC has considered the amendments submitted in response to the Committee's earlier review of your application on 17th June 2002 as set out in our letter dated 25th June 2002. The documents considered were as follows:

- *Patient information sheet (patients with heart failure - physiological stimuli study) version 2 dated 01 July 02*
- *Patient information sheet (patients with heart failure - prevalence study) version 2 dated 01 July 02*
- *Patient information sheet (patients with ischaemic heart disease - physiological stimuli study) version 2 01 July 02*
- *Patient information sheet (patients with ischaemic heart disease - prevalence study) version 2 dated 01 Jul 02*
- *Information sheet (normal subjects-physiologic stimuli) version 2 dated 01 July 02*
- *Information sheet (normal subject: prevalence study) version 2 dated 01 July 02*

The Chair, acting under delegated authority, is satisfied that these accord with the decision of the Committee and has agreed that there is no objection on ethical grounds to the proposed study. I am, therefore, happy to give you the favourable opinion of the committee on the understanding that you will follow the conditions set out below.

Conditions

- You do not undertake this research in an NHS organisation until the relevant NHS management approval has been gained as set out in the Framework *for Research Governance in Health and Social Care.*

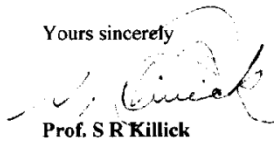
- You do not deviate from, or make changes to, the protocol without prior written approval of the REC, except where this is necessary to eliminate immediate hazards to research participants or when the change involves only logistical or administrative aspects of the research. In such cases the REC should be informed within seven days of the implementation of the change.

- You complete and return the standard progress report form to the REC one-year from the date on this letter and thereafter on an annual basis. This form should also be used to notify the REC when your research is completed and in this case should be sent to this REC within three months of completion.

- If you decided to terminate this research prematurely you send a report to this REC within 15 days, indicating the reason for the early termination.

- You advise the REC of any unusual or unexpected results that raise questions about the safety of the research.

Yours sincerely



Prof. S R Killick
Chair of the Hull and East Riding REC

LREC/ 06/02/117	**Please quote this number on all correspondence**

HULL AND EAST RIDING LOCAL RESEARCH ETHICS COMMITTEE

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Mr M Lammiman
Academic Unit
Department of Cardiology
The Haughton Building
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Anlaby Road
Hull
HU3 2JZ

10 November 2003

Dear Mike,

LREC/ 12/99/191
Protocol number: EECP Enhanced external counterpulsation for angina: a pilot study EECP

Thank you for you recent correspondence dated 24th October 2003 received via email. The Hull and East Riding Local Research Ethics Committee acknowledges receipt of the following documents:

- Revised Patient Information sheet
- Revised Informed Consent form

It is noted that the revisions have been made to remove the name of Dr Louis who has since left the team and also to inform the participant of data protection issues.

The Chair acting under delegated authority has reviewed the above listed documents and is pleased to inform you that the revisions give rise to no ethical issues, he is therefore happy to offer approval on behalf of the committee

Yours sincerely

pp L Coamson

Prof. S R Killick
Chair of the Hull and East Riding REC

LREC/ 12/99/191 Please quote this number on all correspondence

SOUTH HUMBER LOCAL RESEARCH ETHICS COMMITTEE

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SH/KW/04/Q1105/7

5 May 2004

Professor JGF Cleland
Foundation Chair in Cardiology
Academic Department of Cardiology
Castle Hill Hospital
Castle Road
Cottingham
East Yorkshire
HU16 5JQ

Dear Professor Cleland

Full title of study: Enhanced External Counterpulsation (EECP) in patients with ischaemic heart disease and chronic Left Ventricular Systolic dysfunction Evaluation
REC reference number: 04/Q1105/7
Protocol number: Version 1.0 March 2004

The Research Ethics Committee reviewed the above application at the meeting held on 28 April 2004.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion to the above research on the basis described in the application form, protocol and supporting documentation.

The favourable opinion applies to the following research site:

Site: Hull Royal Infirmary - HULL
Principal Investigator: Dr Poay Huan Loh – Clinical Lecturer/Research Fellow

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.

Approved documents

The documents reviewed and approved at the meeting were:

Application Form
Version 3.0
Dated 3.03.04

Investigator CV
Version 1
Dated 17.03.04

Protocol
Version 2
Dated April 2004

Covering Letter
Version 1
Dated 17.03.04

Letters of Invitation of Participants
Version 1
Dated 17.03.04

GP/Consultant Information Sheets
Version 1
Dated 17.03.04

Participant Information Sheet
Version 2
Dated April 2004

Participant Consent Form
Version 1
Dated 17.03.04

Other
Version 1 March 2004
Dated 17.03.04

Management approval

The study may not commence until final management approval has been confirmed by the organisation hosting the research.

You should arrange for all relevant host organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant host organisation before commencing any research procedures. Where a substantive contract is not held with the host organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

SOPs version 1.0 dated February 2004
SL6 Favourable opinion at first review

List of Names and Professions of Members who were Present at the Meeting and those who Submitted Written Comments

Dr Stefan Herber
Consultant/Chairman

Mrs Wendy Witter
Lay Member/Vice-Chairman

Dr Anthony Hill
Director of Public Health

Dr Ian Woollands
GP

Mrs Susan C Clark
Nurse Representative

Mr Jim Hollingworth
Senior Pharmacist

Mrs M Dickerson
Lay Member

Dr R Ezekwesili
Consultant

Mr Stewart Richmond
University Lecturer

Mr Peter Isles
Lay Member

Mr T Bagga
Consultant

Mrs Karen Waltham
LREC Administrator

7.4.2 Assay information

7.4.2.1 D-dimer

Tcoag

TriniLIZE D-Dimer

REF T6005 96 Tests

INTENDED USE

TriniLIZE D-Dimer is intended for the quantitative determination of human D-Dimer in plasma by enzyme immunoassay.

SUMMARY AND PRINCIPLE

The **TriniLIZE D-Dimer** utilises the double antibody principle. Plasma sample or standard containing D-Dimer is added to a microtest well, which is coated with a monoclonal antibody, MA-8D3, against D-Dimer. After an incubation sufficient to allow >85 % of the D-Dimer to bind to the coat antibodies,[7] horse radish peroxidise (HRP) labelled functional antigen-binding (Fab) fragments of anti D-Dimer immunoglobulin G (IgG) are added. These are allowed to react with the adsorbed D-Dimer. The wells are emptied and washed to remove unbound conjugate after which peroxidase substrate (OPD/H_2O_2) is added. The quantity of yellow colour developed is directly proportional to the amount of D-Dimer present in the sample.

REAGENT

For Research Use Only. Not for use in diagnostic procedures.

REAGENT DESCRIPTION

Microtest strips, K3107, 12 Strips
Framed 8-well, pre-coated, with monoclonal antibody MA-8D3, and prefilled with non-immune mouse IgG and indicator dye.
Assay Buffer 15X, K3163, 2 x 33.0 ml
Potassium phosphate, Borate, EDTA, KCl, and Tween 20 buffer solution, pH 7.5 sufficient to make 2 x 0.5 L solution.
Standard 0 ng/mL, K3109, 3 x 0.5 ml
Lyophilised citrated human plasma depleted of D-Dimer.
Standard 1000 ng/mL, K3108, 3 x 0.5 ml
Lyophilised citrated human plasma enriched with D-Dimer.
Conjugate, K3018, 1 x 6.0 ml
HRP labelled Fab fragments of anti D-Dimer antibody.
Substrate, K3105, 1 x 2.0 ml
Lyophilised *ortho*-phenylenediamine (OPD) with buffer salts.
Hydrogen Peroxide, IK267, 1 x 2.0 ml
0.15 % H_2O_2 in purified water.
Reagent Reservoirs, K3087, 6 each
Disposable cardboard trays

REAGENT PREPARATION

Assay Buffer: Dilute the contents of 1 vial up to 0.5 L with purified water.

Standards, 0 and 1000 ng/ml: Add 0.5 ml of Assay buffer to each vial, gently agitate for 5 minutes. Mix 200 µl of 0 ng/ml Standard and 200 µl of 1000 ng/ml Standard in a clean vial to obtain a 500 ng/ml Standard.

Microtest strips: No preparation is required.

Conjugate: Add 6 ml Assay buffer directly to the conjugate vial and agitate gently for 5 minutes.

Substrate concentrate: Add 2 ml of purified water to the vial and agitate until dissolved (10-15 min.).

For one 8-well strip: mix 150 µl of substrate concentrate with 750 µl of purified water and 150 µl of hydrogen peroxide in a clean container to prepare OPD/ H_2O_2 substrate.

For the complete kit: dilute the 2 ml of concentrate with 10 ml of purified water, than add all H_2O_2 to the vial. Invert three times to mix.

Important- The OPD/H_2O_2 substrate should be prepared within 30 minutes of use.

Hydrogen peroxide: Transfer to a clean capped test tube.

ADDITIONAL MATERIALS REQUIRED

- Pipette 8-channel or repeating for 25-100 µl
- Pipettes 1-channel covering 25-1000 µl
- Squeeze bottle
- Paper towels or thin sponge
- Small plastic tubes (2-5 ml)
- Purified water (distilled or deionized and sterile filtered)
- Sulfuric acid H_2SO_4 1.6 mol/L
- Microtest plate spectrophotometer operable at 492 nm
- Microtest plate shaker with an orbital of 3 mm.

MATERIALS AVAILABLE

Microtest strips	Assay Buffer
Standard D-Dimer 0 ng/ml	Standard D-Dimer 1000 ng/ml
Conjugate D-Dimer	Substrate (OPD)
Hydrogen Peroxide	Reagent Reservoirs

STORAGE AND STABILITY

The unopened reagents should be stored at 2-8°C and be used prior to the expiration date.

Assay Buffer: after dilution, store at 2-8°C for two weeks.

Standards 0 and 1000 ng/mL: after reconstitution, store tightly capped at 2-8°C for two days.

Microtest Strips: after breaking the aluminium foil bag, ensure that the remaining strips are sealed tightly in the bag. Store at 2-8°C for two weeks.

Conjugate: after preparation with Assay Buffer, store tightly capped at 2-8°C for two weeks.

Substrate Concentrate: after reconstitution, store in the dark at -20°C for two weeks.

The OPD/H_2O_2 Substrate Concentrate cannot be stored and should be prepared within 30 minutes of use.

Store Hydrogen Peroxide in a clean capped test tube in the dark at 2-8°C for one month.

SPECIMEN COLLECTION AND PREPARATION

Nine volumes of blood are to be collected in one volume of 3.2% (0.109 M) sodium citrate. Immediately after blood collection, samples are centrifuged at 1500 x g for 15 minutes. Please refer to the most recent version of the CLSI document H21 for further instructions regarding specimen collection and storage.[8]

PROCEDURE

WARNINGS AND PRECAUTIONS

The standards are of human origin. Each donor unit of source plasma used in this product has been tested and found negative for HBsAg, anti-HIV I+II, and anti-HCV by FDA-approved methods. However no test can offer complete assurance that products derived from human blood will not transmit infectious disease. As with all materials of human origin, this product should be handled as a potentially infectious agent.

All wastes containing biological material should be properly labelled and stored separately from other wastes. Dispose of all waste materials according to prescribed international, national and local regulations.

Potential carcinogen. The substrate (OPD) and Hydrogen Peroxide are harmful and must be handled with care. Avoid ingestion, skin and eye contact. Wear glasses and gloves when handling.

TEST PROCEDURE

Note: Perform all assay steps at ambient (room) temperature, 18-25°C. Temperature equilibrate all reagent solutions.

1. **Reconstitution of Microtest wells:** Add 50 µl of Assay buffer to each well using a repeating pipette or an 8-channel pipette. Agitate gently for 1 minute.
2. **Standard and sample incubation:** Add 25 µl of D-Dimer standards (0, 500, and 1000 ng/ml) or sample, one addition to each well. The Blue Pre-Fill solution will change colour indicating transfer. Use an air displacement pipette, new tip for each transfer. Record the sample positions. Incubate the strip for 30 minutes on a microtest plate shaker at approximately 600 rpm.
3. **Conjugate incubation:** Add 50 µl of the conjugate to the wells. Use repeating or 8-channel pipette. Incubate the strip for 30 minutes on a micro-test plate shaker at approximately 600 rpm.
4. **Wash:** Discard the contents and wash the strip four times. Each wash is performed as follows; fill the wells completely with Assay buffer, use a squeeze bottle, empty and "dry" by hitting the strip 4-5 times, face down, against absorbing material (sponge, or paper towels).
5. **Substrate incubation:** Add 100 µl of OPD/H_2O_2 substrate to each well. Use repeating or 8-channel pipette. The substrate is prepared within 30 minutes of use. Incubate the strip for 15 minutes on a micro-test plate shaker at approximately 600 rpm.
6. **Stop:** Add 2 ml of concentrated sulfuric acid to 20 ml of purified water. Store in a glass bottle at room temperature. Stop the enzymatic reaction by adding 100 µl of 1.6 mol/L H_2SO_4. Add in the same order and with the same speed as the substrate was added. Agitate the strip for 5 minutes on a micro-test plate shaker to allow complete mixing and stabilisation of colour. If stored in the dark the coloured product is stable for at least 2 hours.
7. **Measurement:** Read the absorbance at 492 nm in a microtest plate spectrophotometer. "Blank" the microtest plate reader against air. Calculations: Plot A_{492} against 0, 500, and 1000 ng/ml. Fit a straight line to the points by a minimal least square procedure, e.g. simple curve fit to Delta Graph® scatter plot. Use the linear function to calculate the D-Dimer values of the samples.

PROCEDURAL NOTES AND PRECAUTIONS

1. Use purified water. Bacterial contamination results in peroxidase activity in the water.
2. Use reagents and strips equilibrated to room temperature to minimise "edge effects", which give rise to erroneous absorbance in the peripheral wells.
3. Make certain the plate shaker does not heat the strips. If the top of the shaker feels warm to the hand, cover with a 1 cm thick sheet of insulation (e.g. Styrofoam®).
4. It is extremely important to remove unbound conjugate before adding the substrate. Be sure that the wash volume completely fills the wells, and that the wells are completely emptied after each wash. Do not leave the empty wells to dry out, fill directly with the next solution.
5. Samples that contain more than 1000 ng/ml D-Dimer should be diluted 1:2 or more with Assay buffer and retested.
6. Some D-Dimer generation may occur during storage in EDTA plasmas, these plasmas can be stored no more than 4 hours at room temperature, 8 hours at 2-8°C or 2 months at -20°C.

QUALITY CONTROL

It is recommended to use a plasma sample containing between 200-400 ng/ml D-Dimer, stored at -20°C, in small aliquots, as a quality control standard each time the assay is run. Failure to obtain D-Dimer levels within two standard deviations of the mean of the control standard may invalidate the assay.

RESULTS

EXPECTED RESULTS

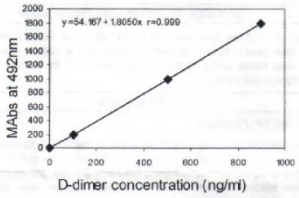
Healthy adults display a mean plasma D-Dimer level of approximately 39 ng/ml.[7] The upper reference limit, 97.5 percentile, is approximately 130 ng/ml.[7] Elevated D-Dimer levels are common in pathological conditions such as disseminated intra-vascular coagulation (DIC), pulmonary embolism (PE), deep venous thrombosis (DVT), pre-eclampsia (pre-EC) and sickle cell crisis.[1-5]

CALCULATION OF RESULTS

Calibration

Plot A_{492} against the quantity of D-Dimer in the standards. Fit a straight line to the points by a minimum least squares procedure. The D-Dimer antigen in the patient's plasma specimen can be determined by interpolation from the standard curve. Note that the slope of the standard curve can show some variation between assays. Users must construct a standard curve each time the assay is performed. See sample standard curve.

Sample Calibration curve



LIMITATIONS

The test is unaffected by rheumatoid factor(s) and antibodies against mouse IgG in the sample. This is due to the use of HRP conjugated Fab fragments as conjugate and a large excess of non-immune mouse IgG in each well. [6]

PERFORMANCE CHARACTERISTICS

Precision

For plasma samples, the intra-assay (within run) and inter-assay (between run) precision is approximately 4% C.V. at 200 ng/ml: at 360 ng/ml both intra- and inter-assay precision is approximately 3% C.V.

Accuracy

The accuracy of the **TriniLIZE D-Dimer** kit was shown in a study of 48 patient plasma samples which were also tested with the semi-quantitative Minutex® D-Dimer latex. A linear regression coefficient of 0.92 was found.

Sensitivity

The test measures D-Dimer antigen in the range 0 to 1000 ng/ml. Maximum sensitivity of the assay is 40 ng/ml sample.

Specificity

The test is specific for D-Dimer by virtue of the screening method used for hybridoma selection.[3] A hybridoma, secreting antibodies that reacted positively with purified D-Dimer but not with whole fibrinogen or fragment D of fibrinogen, was selected. No cross-reactivity with fibrinogen or des-AA-fibrinogen was observed.

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3. Declerck, P.J. *et. al.: Fibrinolytic response and fibrin fragment D-Dimer in patients with deep vein thrombosis.* Thromb. Haemostas. 58: 1025-1029, 1987.
4. Ballegeer, V. et. al.: *Fibrinolytic response to venous occlusion and fibrin fragment D-Dimer levels in normal and complicated pregnancy.* Thromb. Haemostas 58: 1030-1032, 1987.
5. Devine, D.V. *et.al.: Fragment D-Dimer Levels: An Objective Marker of Vaso-occlusive Crisis and Other Complications of Sickle Cell Disease.* Blood. 68 (1): 317-319, 1986.
6. Boscato, L.M. and Stuart, M.C.: *Incidence and specificity of interference in two-site immunoassay* Clin. Chem. 32: 1491-1495, 1986.
7. Rånby, M. and Bergsdorf, N.: *Sandwich ELISA for fibrin Degradation Product D-Dimer Based on Monoclonal Antibody MA-8D3.* Thromb. Haemostas. Abstract #1062 XIVth Congress of the International Society on Thrombosis and Haemostasis, N.Y., 1993.
8. Clinical and Laboratory Standards Institute (CLSI) . *Collection, Transport and Processing of Blood Specimens for Testing Plasma -based Coagulation Assays and Molecular Hemostasis Assays: Approved Guideline – Fifth Edition.* CLSI document H21-A5 Vol. 28. No.5, 2008.

ORDERING INFORMATION

KIT		TriniLIZE D-Dimer
Catalogue No	Item	Quantity
T6005	TriniLIZE D-Dimer	96 Tests

Tcoag Ireland Limited,
IDA Business Park,
Southern Cross Road,
Bray, Co. Wicklow,
Ireland.
Tel. + 353 1 2743200
Fax +.353 1 2746678
www.tcoag.com
info@tcoag.com

T6005 -29 Rev C
02/2011

7.4.2.2 Fibrinogen

ASSAYPRO

AssayMax Human Fibrinogen (FBG) ELISA Kit
(Plasma Samples)

Catalog No. EF1040-1
Lot No. 06251226

Introduction

Fibrinogen (FBG) is a homodimer of molecular mass 340 kDa, made up of two sets of α, β, γ polypeptide chains, and synthesized in the parenchymal cell of the hepatocyte and in the megakaryocyte (1). FBG plays a major role in coagulation, and both elevated and decreased levels have clinical significance. Upon cleavage by thrombin in the initial stages of coagulation activation, FBG self-assembles to yield a fibrin clot matrix that subsequently is crosslinked by factor XIIIa to form an insoluble network. FBG also binds to the platelet glycoprotein IIbIIIa receptor so as to form bridges between platelets, thus facilitating aggregation (2). Elevated plasma FBG has been identified as an independent risk factor for coronary atherosclerosis and ischemic heart disease (3, 4). Individuals with congenital absence of FBG, termed afibrinogenemia, have prolonged bleeding times.

Principal of the Assay

The AssayMax Human Fibrinogen ELISA kit is designed for detection of human FBG in plasma. This assay employs a quantitative competitive enzyme immunoassay technique that measures FBG in less than 3 hours. A murine antibody specific for FBG has been pre-coated onto a 96-well microplate with removable strips. FBG in standards and samples is competed with a biotinylated FBG sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-protein, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution

Reagents

- **FBG Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a murine antibody against FBG.

- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **FBG Standard:** Human FBG in a buffered protein base (120 µg, lyophilized).
- **Biotinylated FBG:** 1 vial, lyophilized.
- **MIX Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate)**: A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard and Biotinylated Protein at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000µl and multiple channel pipettes).
- Deionized or distilled reagent grade water.

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and use supernatants for assay. Dilute samples 1: 2000 into MIX Diluent. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA can also be used as anticoagulant).

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 120 µg of FBG Standard with 3 ml of MIX Diluent to generate a stock solution of 40 µg/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard solution (40 µg/ml) 1:3 with MIX Diluent to produce 13.33, 4.44, 1.48, 0.49, and 0.16 µg/ml solutions. MIX Diluent serves as the zero standard (0 µg/ml). Any remaining solution should be frozen at -20^{0}C and used within 30 days.

Standard Point	Dilution	[FBG] (µg/ml)
P1	1 part Standard (40 µg/ml)	40.00
P2	1 part P1 + 2 parts MIX Diluent	13.33
P3	1 part P2 + 2 parts MIX Diluent	4.444
P4	1 part P3 + 2 parts MIX Diluent	1.481
P5	1 part P4 + 2 parts MIX Diluent	0.494
P6	1 part P5 + 2 parts MIX Diluent	0.165
P7	MIX Diluent	0.000

- **Biotinylated FBG (2x):** Dilute Biotinylated FBG with 4 ml MIX Diluent to produce a 2-fold stock solution. Allow the biotin to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:2 with MIX diluent. Any remaining solution should be frozen at -20^{0}C and used within 30 days.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 25 µl of standard or sample per well and immediately add 25 µl of Biotinylated FBG to each well (on top of the Standard or sample). Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for about 8 minutes or till the optimal color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

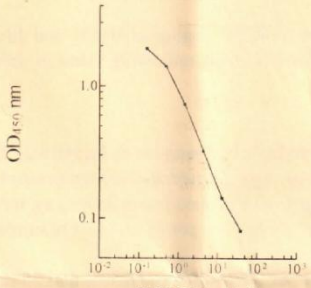
Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot 4-parameter graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. T The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

Human FBG
Standard Curve



Performance Characteristics

- The minimum detectable dose of FBG is typically ~0.16 µg/ml.
- Intra-assay and inter-assay coefficients of variation were 4.9 % and 7.5% respectively.
- FBG standard in this kit has been calibrated against WHO reference plasma.

Linearity

	Average Percentage of Expected Value
Sample Dilution	**Plasma**
1:1000	92%
1:2000	101%
1:4000	105%

Recovery

Standard Added Value	1 - 10 µg/ml
Recovery %	86-111 %
Average Recovery %	98.5 %

Reference Value

- Normal human plasma FBG concentration has been reported ranging approximately 2.26 to 3.3 mg/ml (5).

Cross-Reactivity

Species	% Cross Reactivity
Canine	None
Bovine	None
Monkey	0.5%
Mouse	None
Rat	0.01%
Swine	0.5%
Rabbit	0.01%
Human	100%

References

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(3) Handa, K. *et al.* (1989) *Atherosclerosis* 77:209
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Related Products

- EF2040-1 AssayMax Human Fibrinogen ELISA Kit (Urine, Milk, Saliva and Cell Culture Supernatant samples)
- ERF2040-1 AssayMax Rat Fibrinogen ELISA Kit (Urine and Cell Culture Supernatant samples)
- ERF1040-1 AssayMax Rat Fibrinogen ELISA Kit (Plasma samples)
- EMF2040-1 AssayMax Mouse Fibrinogen ELISA Kit (Urine and Cell Culture Supernatant samples)
- EMF1040-1 AssayMax Mouse Fibrinogen ELISA Kit (Plasma samples)

7.4.2.3 von Willebrand Factor activity

ENGLISH

REAADS®
VON WILLEBRAND FACTOR ANTIGEN TEST KIT

For *In Vitro* Diagnostic Use

INTENDED USE

An enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of Von Willebrand Factor Antigen (VWF:Ag) in citrated human plasma.

SUMMARY AND EXPLANATION OF THE TEST

Von Willebrand Factor Antigen (VWF:Ag or Factor VIII-related protein) is a plasma protein found in circulation combined by non-covalent interactions with Factor VIII (FVIII:C), a pro-coagulant protein also known as the anti-hemophilic factor. These two proteins show distinct biochemical and functional properties as well as different antigenic determinants; their plasma levels may vary independently of each other.[1,2] Deficiency of FVIII causes classic hemophilia while deficiency of VWF causes Von Willebrand disease. Most of VWF:Ag is synthesized and stored by endothelial cells while 15-20% is synthesized by megakaryocytes and stored in circulating platelets. A VWF:Ag unit has a molecular weight of about 250 kD and tends to polymerize in circulation, with multimers ranging in size from 850kD to as large as $15x10^6$ D.[3]

VWF:Ag plays a very important role in hemostasis; it protects FVIII from proteolytic cleavage in circulation and helps platelets to aggregate or to adhere to sites of vascular damage. The in vivo half-life of FVIII:C without VWF:Ag is shortened from 10-12 hours to a few minutes. These two mechanisms prevent bleeding. Von Willebrand disease is characterized by an inherited deficiency of VWF. A decreased VWF activity in plasma can be the result of low concentrations (quantitative or type I defect) or a deficient function of VWF (qualitative or type II defect).[4,5] Von Willebrand disease is the most common inherited bleeding disorder characterized by easy bruising and prolonged bleeding from mucosal surfaces. The prevalence of Von Willebrand disease has been estimated to be 1-3% of the general population. Approximately 80% of Von Willebrand disease patients have a type I deficiency.[6]

The laboratory diagnosis of Von Willebrand disease may require both quantitative and qualitative (functional) determinations.[7,8] Quantitative determinations are based on immunologic techniques such as radial immunodiffusion in gel and Laurell rocket immunoelectrophoresis. ELISA procedures[9] applied to measure VWF:Ag are less labor intensive and offer several advantages including more objective, accurate and reproducible results. In addition, ELISA allows automation with commonly available laboratory instruments.

PRINCIPLE OF THE TEST

REAADS VWF:Ag assay is a sandwich ELISA. A capture antibody specific for human VWF is coated to 96-microwell polystyrene plates. Diluted patient plasma is incubated in the wells, allowing any available VWF:Ag to bind to the anti-human VWF antibody on the microwell surface. The plates are washed to remove unbound proteins and other plasma molecules. Bound VWF:Ag is quantitated using horseradish peroxidase (HRP) conjugated anti-human VWF detection antibody. Following incubation, unbound conjugate is removed by washing. A chromogenic substrate of tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) is added to develop a colored reaction. The intensity of the color is measured in optical density (O.D.) units with a spectrophotometer at 450nm. Patient VWF:Ag in relative percent concentration is determined against a curve made from the reference plasma provided with the kit.

1

REAGENTS

Store at 2 - 8°C. Do Not Freeze.

Each READS Von Willebrand Factor Antigen (VWF:Ag) 96-microwell Test Kit contains the following reagents:

- 12 x 8 anti-human Von Willebrand Factor antibody coated microwells.
- 60 mL Sample Diluent (blue-green solution); contains sodium azide.
- 3 x 0.5 mL lyophilized Reference Plasma, with assay sheet.
- 12 mL anti-human VWF HRP Conjugate (red solution).
- 13 mL Substrate (TMB and H_2O_2).
- 15 mL Stopping Solution (0.36 N sulfuric acid).
- 30 mL Wash Concentrate (33X phosphate buffered saline with 0.01% Tween 20). Note: turbidity may appear in wash concentrate which will not affect component performance and should disappear when working dilution is prepared.

WARNINGS AND PRECAUTIONS

For *In Vitro* Diagnostic Use

1. Human source material used to prepare the reference plasma included in this kit has been tested and shown to be negative for antibodies to HBsAg, HCV and HIV-I & II by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material.
2. Do not pipette by mouth.
3. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
4. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
5. One-component substrate can cause irritation to the eyes and skin. Absorption through the skin is possible. Use gloves when handling substrate and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.
6. Certain components are labeled with the following:
   Irritating to eyes (R 36). Avoid contact with skin (S 24). Avoid contact with eyes (S 25). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S 26). If swallowed, seek medical advice immediately and show this container or label (S 46).

   Irritant. Biological Risk.

SPECIMEN COLLECTION AND PREPARATION

Plasma collected with either 3.2% or 3.8% sodium citrate as an anticoagulant should be used as the sample matrix. Blood should be collected by venipuncture, and the sample centrifuged immediately. Remove the plasma and store at 2 - 8°C until testing can be performed. If not tested within 8 hours of collection, the plasma sample should be stored at -70°C and tested within 1 month.

INSTRUCTIONS FOR USE

Materials Provided

READS Von Willebrand Factor Antigen Test Kit; see "Reagents," for a complete listing.

Materials Required but not Supplied

- VWF:Ag Control Plasma. Follow manufacturer's instructions, and store as recommended.
- Reagent grade water (1L) to prepare PBS/Tween 20 wash solution, to reconstitute Reference Plasma, and to zero or blank the plate reader during the final assay step
- Graduated cylinders
- Precision pipettors capable of delivering between 5 and 1000 µL, with appropriate tips

- Miscellaneous glassware appropriate for small volume handling
- Flask or bottle, 1 liter
- Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated washing system
- Disposable gloves, powder-free recommended
- Plate reading spectrophotometer capable of reading absorbance at 450nm (with a 650nm reference if available)
- Multichannel pipettors capable of delivering to 8 wells simultaneously
- Microdilution tubes for patient sample preparation

Procedural Notes

1. Bring plasma samples and kit reagents to room temperature (18 - 26°C) and mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated storage (2 - 8°C) as soon as possible.
2. All dilutions of reference plasma, control plasma selected for use, and patient samples must be made just prior to use in the assay.
3. A single water blank well should be set up on each plate with each run. No sample or kit reagents are to be added to this well. Instead, add 200 µL of reagent grade water to the well immediately prior to reading the plate in the spectrophotometer. The plate reader should be programmed to zero or blank against this water well.
4. Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution from a plastic squeeze bottle with a wide tip into the bottom of the microwells. Wash solution in the water blank well will not interfere with the procedure. An automated microtiter plate washing system can also be used.
5. IMPORTANT: Failure to adequately remove residual PBS/Tween 20 can cause inconsistent color development of the substrate solution.
6. Use a multichannel pipettor capable of delivering to 8 wells simultaneously when possible. This speeds the process and allows for more uniform incubation and reaction times for all wells.
7. Carefully controlled timing of all steps is critical. All reference plasma dilutions, controls and samples must be added to the microwells within a five minute period. Batch size of samples should not be larger than the amount that can be added within this time period.
8. For all incubations, the start of the incubation period begins upon the completion of reagent or sample addition.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures above or below normal room temperature (18 - 26°C) may contribute to inaccurate results.
11. Avoid contamination of reagents when opening and removing aliquots from the primary vials.
12. Do not use kit components beyond expiration date.
13. Coated microwells, conjugate, and substrate are lot specific components that should not be used with different kit lots.

Reagent Preparation

1. Wash Solution - phosphate buffered saline (PBS)/Tween 20: Measure 30 mL Wash Concentrate (33X PBS/Tween 20) and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1. Store unused PBS/Tween 20 solution at 2 - 8°C. Discard if solution shows signs of contamination.
2. Reconstitute Reference Plasma by adding 0.5 mL reagent grade water. Swirl gently to mix. Allow to stand for 10 minutes before use for complete dissolution. Stable for 8 hours when stored at 2 - 8°C. Reconstitute appropriate control plasma following manufacturer's instructions, and store as recommended.

Assay Procedure

1. Remove any microwell strips that will not be used from the frame and store them in the bag provided.
2. Assay each reference plasma dilution in duplicate. Duplicate determinations are also recommended for patient and control samples. One well should be run as a reagent blank; sample diluent without plasma is added to the well as explained in step 6 of this section. This well is treated the same as a patient sample in subsequent assay steps. A water blank well should be included with each plate; it is to remain empty until 200 µL of reagent grade water is added at the completion of the assay, immediately prior to reading the plate. The water blank well is to be used to zero the plate reader.
3. Using the Reference Plasma provided with the kit, prepare six reference plasma dilutions as described below:

Volume Reference Plasma		Volume Sample Diluent		*Reference Level (%)
30 µL	+	500 µL	=	150
20 µL	+	500 µL	=	100
15 µL	+	500 µL	=	75
10 µL	+	500 µL	=	50
10 µL	+	1000 µL	=	25
10 µL	+	2000 µL	=	12.5
**10 µL	+	**4000 µL	=	6.25

* Reference level value to be used for constructing reference curve only.
**Make one additional dilution if the assayed value of the Reference Plasma is ≥150%.

4. Prepare a 1:26 dilution of each patient sample and control plasma selected for use in Sample Diluent (blue-green solution); e.g. 20 µL sample added to 500 µL Sample Diluent. Mix thoroughly.
5. Add 100 µL of the dilutions (reference plasmas x 6, patient samples and controls) to the appropriate microwells.
6. Add 100 µL of Sample Diluent to the reagent blank well. Leave the water blank well empty.
7. Incubate 15 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the fluid. Do not allow samples to contaminate other microwells.
8. Wash 4 times with working wash solution (PBS/Tween 20). Each well should be filled with wash solution per wash. Wash solution in the empty well intended to serve as a water blank will not interfere with the procedure. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. The frame must be squeezed at the center on the top and bottom to retain microwell modules during washing. Blot on absorbent paper to remove residual wash fluid. Do not allow wells to dry out between steps.
9. Add 100 µL Conjugate (red) to each well (except the water blank well).
10. Incubate for 15 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the conjugate solution.
11. Wash 4 times with working wash solution (PBS/Tween 20) as in step 8. Wash solution in the water blank well will not interfere with the procedure. Use a snapping motion to drain the liquid, and blot on absorbent paper after the final wash. Do not allow the wells to dry out.
12. Add 100 µL Substrate to each well (except for the water blank well) and incubate for 10 minutes at room temperature. Add the substrate to the wells at a steady rate. Blue color will develop in wells with positive samples.
13. Add 100 µL Stopping Solution (0.36 N sulfuric acid) to each well (except for the water blank well) to stop the enzyme reaction. Be sure to add Stopping Solution to the wells in the same order and at the same rate as the Substrate Solution was added. Blue substrate will turn yellow and colorless substrate will remain colorless. Do not add Stopping Solution to the water blank well. Instead, add 200 µL reagent grade water to the water blank well. Blank or zero the plate reader against the water blank well. Read the O.D. of each well at 450nm, against a 650nm reference filter (if available). For best results, the O.D. values should be measured within 30 minutes after the addition of Stopping Solution.

RESULTS

1. Calculate the mean O.D. values for the duplicates of the reference plasma dilutions, controls selected for use, and patient samples.
2. Plot the mean O.D. obtained for each dilution of the reference plasma (x axis) against the corresponding value of the reference level (y axis). The curve may be plotted on a linear, semi-log or log-log graph. Draw a line to connect the points.
3. Using the mean O.D., determine the control and patient relative values from the graph, or alternatively, use linear regression to calculate from the reference curve.
4. To calculate VWF:Ag levels in percent (%) of normal, multiply the control and patient relative values (obtained from the reference curve) by the assigned value for the REAADS Reference Plasma (see vial label).
   For example:
   Patient relative value (from the reference curve): 40
   Reference Plasma assigned value (from vial label): 105% of normal
   Actual patient VWF:Ag value (as % of normal): 40 x 1.05 = 42%
5. Ensure that all quality control parameters have been met (see Quality Control) before reporting test results.

QUALITY CONTROL

1. The mean O.D. of the reagent blank should be less that 0.1 when the spectrophotometer has been blanked against the water well. Readings greater than 0.1 may indicate possible reagent contamination or inadequate plate washing.
2. O.D. values for the duplicates of the controls or patient samples should be within 20% of the mean O.D. value for samples with absorbance readings greater than 0.200.
3. VWF:Ag values obtained for the controls should fall within manufacturer's assigned ELISA ranges. Occasional small deviations outside these ranges may be acceptable.
4. Each laboratory should periodically determine their own reference range for this assay.

EXPECTED VALUES[10]

Normal Range: Plasma VWF:Ag values are generally expressed in relative percent (%) as compared to pooled normal plasma. The normal range when normal plasma samples were tested by REAADS VWF:Ag assay was 47 - 197% (mean 105.8%, SD 39%). This range is consistent with that published in the literature[4,7] and reported by other commercially available assays (50-160%). Samples with values above the range of the reference curve may be diluted and retested for accurate results.

PERFORMANCE CHARACTERISTICS[10]

Detection range:

The detection range for REAADS VWF:Ag assay has been determined to be 5 - 200%. However, the effective range of each run will depend on the assayed value of the reference plasma. For greatest accuracy, samples which generate absorbance readings outside the O.D. range of the reference curve should be retested at an appropriate dilution.

Precision:

Intra-assay precision:

To determine variability within a plate, three plasma samples with known VWF levels (one high, one medium, and one low) were tested in 16 wells by two operators, on six plates from each of three lots. The data, presented in the following table, shows a mean CV of 3.6% across three lots. In addition, ninety-nine (99) patient samples with VWF levels ranging from 54 - 276% of normal were tested in duplicate across 3 lots to demonstrate the precision end users may expect when performing the assay according to package insert instructions. As shown in the table, the overall mean CV for duplicates was 2.5%.

Reference Plasma

Assay Sheet - ELISA Values

LOT **2-10-551654***

2013-09-30

Product	Value
Von Willebrand Factor Antigen =	92%
Von Willebrand Factor Activity =	86%
Protein C Antigen =	104%
Total Protein S Antigen =	98%
Free Protein S Antigen by PEG Precipitation =	94%
Monoclonal Free Protein S Antigen =	110%

*The alpha character in the vial lot number is for internal use only; It is not shown on this Value Assignment Sheet.

61-017-1 19
Effective: 2008-07-01

Inter-assay precision:

Ten (10) commercially prepared, assayed plasma samples with VWF values ranging from 57 - 159% were tested in duplicate on three lots to determine assay precision between lots. The mean inter-assay CV was 5%, as seen in the table:

	VWF range (% of normal)	CV Range (3 pilot lots)	Overall mean CV:
Intra-assay precision (variability within a plate)			
Replicates (x16):	149% - 155%	1.9 - 7.9%	3.6%
	75% - 89%	2.2 - 7.7%	
	57% - 83%	1.8 - 9.9%	
Duplicates:	54% - 276%		2.5%
Inter-assay precision (variability between lots) Duplicates:	57% - 159%	3.0 - 12.1%	5.0%

Linearity:

Serial two-fold dilutions of VWF reference plasma samples tested on three lots of REAADS VWF:Ag assay demonstrated curves with a mean coefficient of determination (R squared) of 0.995; individual point recovery ranged from -10.7% to +14.0%.

Accuracy:

Accuracy was determined by testing mixtures of reference plasma with predetermined values on REAADS VWF:Ag assay and calculating the recovery of theoretical values. The overall mean percent recovery across 3 lots was 103.6% with an average variation of 5.7%.

LIMITATIONS OF THE TEST

The VWF:Ag levels obtained from this assay are an aid to diagnosis only. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures. There is a normal plasma fluctuation of VWF:Ag due to unknown mechanisms. For this reason, repeat testing may be necessary. In addition, VWF:Ag acts as an acute phase reactant; it may be increased in various stressful conditions and diseases including pregnancy, oral contraceptive use, surgery, liver and autoimmune diseases, prostate cancer, etc.[4,6]

Plasma samples can be inadvertently depleted or degraded of VWF:Ag by improper collection or laboratory processing. Individuals with "O" blood type have been shown to have lower plasma levels of VWF:Ag (≈ 25%) when compared to those with other blood types. Acquired Von Willebrand disease has been reported in some patients with lymphoproliferative disease.[7]

As with any assay employing antibodies from an animal source (e.g. mouse, rabbit, goat, etc.) to capture a target molecule, the possibility exists for interference in the serum or plasma of patients who have been exposed to preparations containing animal antibodies for diagnosis or therapy. Falsely elevated or depressed values may be seen in these patients.

WARRANTY

This product is warranted to perform as described in this package insert. Corgenix, Inc. disclaims any implied warranty of merchantability or fitness for a particular use, and in no event shall Corgenix, Inc. be liable for consequential damage.

For Technical or Customer Service in the United States, phone 1-800-729-5661. Outside the United States, phone (303) 457-4345, fax (303) 457-4519, e-mail: technicalsupport@ corgenix.com, or contact a Corgenix authorized distributor.

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7.4.2.4 Heart-type fatty acid-binding protein

1. INTENDED USE

The human H-FABP ELISA kit is to be used for the *in vitro* quantitative determination of human H-FABP in serum or plasma samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The kit is presented in a two assay format. The normal format takes about 1½ hours. The rapid format takes about 45 minutes.

The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

Fatty acid-binding proteins (FABPs) are a class of cytoplasmic proteins that bind long chain fatty acids. FABPs are small intracellular proteins (~13-14 kDa) with a high degree of tissue specificity. They are abundantly present in various cell types and play an important role in the intracellular utilization of fatty acids, transport and metabolism. There are at least nine distinct types of FABP, each showing a specific pattern of tissue expression. Due to its small size, FABP leaks rapidly out of ischemically damaged necrotic cells leading to a rise in serum levels. Ischemically damaged tissues are characterized histologically by absence (or low presence) of FABP facilitating recognition of such areas.

Following acute myocardial infarction (AMI) the small protein H-FABP is rapidly released into the circulation. H-FABP is derived from the human *FABP3* gene. Significantly elevated serum/plasma concentrations are found within 3 h after AMI which generally return to normal values within 12 to 24 h. These features make H-FABP a useful research tool for the early assessment or exclusion of AMI, and for the monitoring of a recurrent infarction. Constitutive H-FABP released from the heart after AMI is quantitatively recovered in serum/plasma. Thus assessment of H-FABP is also a very effective tool for the estimation of the infarct size. The human H-FABP kit can also be used for measurement of brain-type FABP, a marker for brain injury detection and for measurement of muscle-type cytosolic fatty acid binding protein (FABPc) in skeletal muscle.

In serum/plasma of healthy individuals approximately 1.6 ng/ml of H-FABP is present. H-FABP shows a slight increase with age.

3. KIT FEATURES

- Working time of 1½ hours (normal) or ¾ (rapid) hour.
- Minimum concentration which can be measured is 102 pg/ml.
- Measurable concentration range of 102 to 25,000 pg/ml.
- Working volume of 100 µl/well.

Cross-reactivity

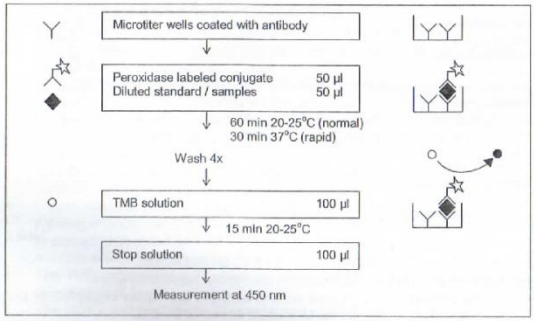
Potential cross-reacting proteins detected in the human H-FABP ELISA:

Cross reactant	Reactivity
Human I-FABP	Negative
Human L-FABP	Negative
Swine H-FABP	Average
Horse H-FABP	Average
Salmon H-FABP	Average

Table 1

Cross-reactivity for other proteins/peptides has not been tested.

4. PROTOCOL OVERVIEW



- The human H-FABP ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 1½ (normal) or ¾ (rapid) hours.
- The efficient format of 2 plates with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples and standards are incubated together with peroxidase-conjugated second antibody in microtiter wells coated with antibodies recognizing human H-FABP.
- During incubation human H-FABP is captured by the solid bound antibody. The secondary antibodies will bind to the captured human H-FABP.
- The peroxidase-conjugated second antibody will react with the substrate, tetramethylbenzidine (TMB).
- The enzyme reaction is stopped by the addition of oxalic acid.
- The absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human H-FABP standards (log).
- The human H-FABP concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Item no.	Kit component	Quantity	Color code
Vial 1	Wash buffer 20x	1 vial (20 ml)	Grey
Vial 2	Dilution buffer 10x	1 vial (10 ml)	Gold
Vial 3	Standard	1 vial, 1 ml lyophilized	Yellow
Vial 4	Conjugate, peroxidase-labeled	2 vials, 1 ml lyophilized	Green
Vial 5	TMB substrate	1 vial (20 ml)	Purple
Vial 6	Stop solution	1 vial (20 ml)	Red
Item 7	12 Microtiter strips, pre-coated	2 plates	
Item 8	Frame	1	
Item 9	Adhesive covers	4	
Item 10	Certificate of analysis	1	
Item 11	Manual	1	
Item 12	Data collection sheet	1	

Table 2

- Upon receipt, store individual components at 2 - 8°C. Do not freeze.
- Do not use components beyond the expiration date printed on the kit label.
- The standard and peroxidase-conjugated second antibody are stable in lyophilized form until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The exact concentration of the standard is indicated on the label of the vial and the certificate of analysis.
- Once reconstituted, standard and peroxidase-conjugated second antibody are stable for 1 month if stored at 2 - 8°C.
- Upon receipt, foil pouch around the plate should be vacuum-sealed and unpunctured. Any irregularities to aforementioned conditions may influence plate performance in the assay.
- Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality guaranteed until expiration date if stored at 2 - 8°C.

Materials required but not provided

- Calibrated micropipettes and disposable tips.
- Distilled or de-ionized water.
- Plate washer: automatic or manual.
  In case a plate washer is used the supplied wash buffer is not sufficient. Additional wash buffer can be ordered separately. Please contact your local distributor.
- Polypropylene tubes.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.

6. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not under any circumstances add sodium azide as preservative to any of the components.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by manufacturer.
- The assay has been optimized for the indicated standard range. Do not change the standard range.
- Standard and conjugate vials should be opened after reconstitution. Open vials carefully: vials are under vacuum.
- Do not ingest any of the kit components.
- Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
- The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
- The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advice.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.
- Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.

7. SAMPLE PREPARATION

Collection and handling

Serum or plasma

Collect blood using normal aseptic techniques. If serum is used, separate serum from blood after clotting at room temperature within 1 hour by centrifugation (1500xg at 4°C for 15 min). Transfer the serum to a fresh polypropylene tube.

If plasma is used, separate plasma from blood within 20 minutes after blood sampling by centrifugation (1500xg at 4°C for 15 min). Transfer the plasma to a fresh polypropylene tube.

Most reliable results are obtained if heparin or EDTA plasma is used.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of human H-FABP. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of human H-FABP activity and give erroneous results.

Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Serum or plasma samples

Human H-FABP can be measured accurately if serum or plasma samples are diluted at least 5x with supplied dilution buffer. in polypropylene tubes

Most reliable results are obtained if heparin or EDTA plasma is used.

Remark regarding recommended sample dilution

The recommend dilution for samples should be used as a guideline. The recovery of human H-FABP from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human H-FABP.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

50/200

8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use.

2) **Wash buffer**

Prepare wash buffer by mixing 20 ml of 20x wash buffer with 380 ml of distilled or de-ionized water, which is enough for 2 x 96 tests. Where less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 20x wash buffer with 19 parts of distilled or de-ionized water.

-500ml

1) **Dilution buffer**

Prepare dilution buffer by mixing 10 ml of the 10x dilution buffer with 90 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. Where less volume is required, prepare the desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer with 9 parts of distilled or de-ionized water. Concentrated dilution buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37°C. Do not shake the solution.

Standard solution

The standard is reconstituted by injection of 1 ml of distilled or de-ionized water. Prepare each human H-FABP standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Figure 1.

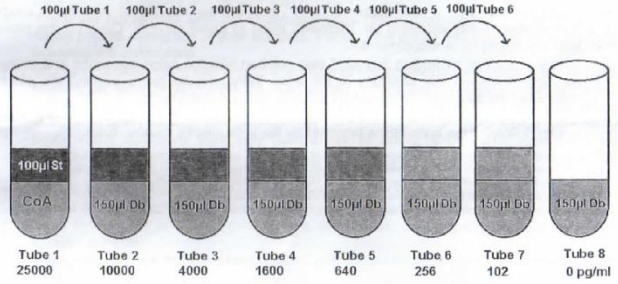


Figure 1

Conjugate solution

The peroxidase-conjugated second antibody is reconstituted by injection of 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml Conjugate with 5 ml dilution buffer, which is sufficient for 1 x 96 tests. Where less volume is required, prepare the desired volume of conjugate by diluting 1 part of the reconstituted Conjugate with 5 parts of dilution buffer.

neg control do not add

9. ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.
2. Add 50 µl of diluted peroxidase-conjugated second antibody to each well.
3. Transfer 50 µl in duplicate of standard, samples, or controls into appropriate wells.
4. Apply an adhesive cover to the tray. Tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
5. Incubate the strips or plate for 60 minutes at room temperature for normal format or 30 mintutes at 37°C for rapid format.
6. Wash the plates 4 times with wash buffer using a plate washer or as follows*:
   a. Carefully remove the plate sealer, avoid splashing.
   b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
   c. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate as described in 6b.
   d. Repeat the washing procedure 6b/6c three times.
   e. Empty the plate and gently tap on thick layer of tissues.
7. Add 100 µl of TMB substrate to each well. Do not touch the side or bottom of the wells.
8. Cover the tray with a new adhesive cover, incubate the tray for 15 minutes at room temperature. Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminium foil is recommended.
9. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 7. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
10. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

*) In case plate washer is used, please note: use of a plate washer can result in higher background and decrease in sensitivity. We advise validation of the plate washer with the manual procedure. Make sure the plate washer is used as specified for the manual method. Additional wash buffer can be ordered separately. Please contact your local distributor.

10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.3.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

11. TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.
- If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
- Since exact conditions may vary from assay to assay, a standard curve must be established for every run. If the standard is out of range, the results of the test samples are not reliable. The test should be repeated.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, sample, Conjugate and buffers should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of supplied covers during incubation steps is necessary.
- The waste disposal should be performed according to your laboratory regulations.

Technical support

Do not hesitate to contact our technical support team at support@hycultbiotech.com for inquiries and technical support regarding the human H-FABP ELISA.

Hycult Biotech, Frontstraat 2a, 5405 PB Uden, the Netherlands
T: +31 (0)413 251 335, F: +31 (0)413 248 353

12. QUALITY CONTROL

The certificate of analysis included in this kit is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the certificate of analysis are to be used as a guideline only. The results obtained by your laboratory may differ.

This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Hycult Biotech immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advised to work according to good laboratory practice.

13. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before expiry date of the product. A written complaint containing lot number of the product and experimental data should sent to support@hycultbiotech.com.

Suggestions summarized below in Table 33 can be used as a guideline in the case of unexpected assay results.

Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
•	•		•	•	Kit materials or reagents are contaminated or expired
•					Incorrect reagents used
•		•	•		Lyophilized reagents are not properly reconstituted
•	•	•	•	•	Incorrect dilutions or pipetting errors
•		•			Improper plastics used for preparation of standard and/or samples
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•					Assay performed before reagents were brought to room temperature
•	•	•	•	•	Procedure not followed correctly
				•	Omission of a reagent or a step
		•			Poor mixing of samples
	•		•		Low purity of water
	•	•			Strips were kept dry for too long during/after washing
	•	•	•		Inefficient washing
	•	•			Cross-contamination from other samples or positive control
		•	•		TMB solution is not clear or colorless
•	•				Wrong filter in the microtiter reader
	•	•			Airbubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions

Table 3

14. REFERENCES

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7.5 Definitions

Abbreviations

ACEI	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndrome
ADHF	Acute decompensated heart failure
ARA	Aldosterone receptor antagonist
ARB	Angiotensin receptor blocker
AUC	Area under the curve
BB	β-blocker
BNP	B-typre natriuretic peptide
CABG	Coronary artery bypass graft surgery
CAD	Coronary artery disease
CCS	Canadian Cardiovascular Society angina grading
CHF	Chronic heart failure
CI	Cardiac index
CMR	Cardiac magnetic resonance imaging
CO	Cardiac output
DASI	Duke's Activity Status Activity
DD	D-dimer
ECG	Electrocardiogram
EECP	Enhanced external counterpulsation
EPO	Erythropoietin
FBC	Full blood count
FBG	Fibrinogen
GTN	Glyceryl trinitrate

GFR	Glomerular filtration rate
H-FABP	Heart-type faty acid-binding protein
HF	Heart failure
HR	Hazard ratio
hs-CRP	High-sensitivity c-reactive protein
hs-TnT	High-sensitivity troponin T
IEPR	International EECP Patient Registry
IHD	Ischaemic heart disease
KCCQ	Kansas City Cardiomyopathy Questionnaire
LV	Left ventricle
LVEDD	LV end diastolic diameter
LVEDP	LV end diastolic pressure
LVEDV	LV end diastolic volume
LVEF	LV ejection fraction
LVESD	LV end systolic diameter
LVESV	LV end systolic volume
MACE	Major adverse cardiovascular event
MI	Myocardial infarction
MLHFQ	Minnesota Living with Heart Failure Questionnaire
NLR	Neutrophil-to-lymphocyte ratio
NEC	Neutrophil count
NSTEMI	Non-STEMI
NT-proBNP	Amino acid terminal of precursor of BNP
NYHA	New York Heart Association breathlessness severity
OR	Odd ratio

pVO_2	Peak oxygen uptake
PAI-	Plasminogen activator inhibitor-1
PCI	Percutaneous coronary intervention
RAP	Right atrial pressure
RDW	Red cell distribution width
RLC	Relative lymphocyte count
RNC	Relative neutrophil count
ROC	Receiver operator characteristic
STEMI	ST segment elevation myocardial infarction
SV	Stroke volume
t-PA	Tissue plasminogen activator
vWF	von Willebrand factor
WCC	White cell count