

# Natriuretic Peptides and the Microcirculation in Heart Failure Patients

Marie-Louise Edvinsson



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DOCTORAL THESIS

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**Faculty Opponent**

Professor *Kurt Boman*, Umeå University

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| Title and subtitle: Natriuretic peptides and the microcirculation in heart failure patients   |                                      |                        |
| <p>Abstract: The increased prevalence of chronic congestive heart failure (HF) is a tremendous challenge for society. In spite of effective medical treatment for acute HF, better pharmacological treatment for specific neurohormonal intervention and better diagnostic tools, e.g. biomarkers of natriuretic peptides, the morbidity and mortality due to this disease are still prominent. HF leads to vascular dysfunction in the general circulation but the interpretation of this is not clear. In our studies we have focussed on defining endothelial and smooth muscle dysfunction in the microvasculature of HF patients and correlating this dysfunction with the disease state.</p> <p>This thesis addresses studies of elderly patients with varying degrees of HF. We determined blood levels of homocysteine and the natriuretic peptide precursor, NT-proBNP which is released by the failing heart. We also investigated vasoreactive response of the cutaneous microcirculation to different stimuli using a non-invasive iontophoresis-Laser Doppler probe method.</p> <p>Paper I: We investigated the prognostic value of NT-proBNP for monitoring progression of HF. High blood levels (&gt;5000) were found to indicate a poor prognosis of HF.</p> <p>Paper II: An open study,- in which we evaluated the function of the cutaneous peripheral circulation of patients with chronic HF. We found that vasoreactivity declines with increasing age and HF.</p> <p>Paper III: We evaluated vasoreactive responses in HF patients with homocysteinemi, before and 6 weeks after supplementary by B vitamin treatment. Homocysteine levels where then normalized and the cutaneous responses improved.</p> <p>Paper IV: A controlled study in which, we investigated the microcirculation in chronic HF patients with different degrees of HF. We saw that the degree of severity of congestive HF did not correlate with decrease in vasoreactive responses.</p> <p>Paper V: The effect of BNP in the microcirculation was studied in patients with severe congestive HF and compared to healthy, matched controls. The result shows that BNP has a significantly weaker vasodilation in HF patients and this is probably due to down regulation at the receptor-coupling level.</p> |                                      |                        |
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Cover illustration:

The picture of a human vessel, shaped in a form of a heart, purely by random.

Photo by: Karin Warfvinge

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LUND UNIVERSITY  
Faculty of Medicine  
Department of Clinical Sciences, Lund  
Department of Emergency Medicine

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*Det anstår mig inte att göra  
mig mindre än jag är*

Edith Södergran, 1918



# Original Articles

- I. High NT-proBNP is a strong predictor of outcome in elderly heart failure patients. Andersson SE, **Edvinsson ML**, Björk J, Edvinsson L. *Am J Geriatr Cardiol*. 2008 Jan-Feb; 17(1):13-20.
- II. Cutaneous vascular reactivity is reduced in aging and in heart failure: association with inflammation. Andersson SE, **Edvinsson ML**, Edvinsson L. *Clin Sci (Lond)*. 2003 Dec; 105(6):699-707.
- III. Reduction of homocysteine in elderly with heart failure improved vascular function and blood pressure control but did not affect inflammatory activity. Andersson SE, **Edvinsson ML**, Edvinsson L. *Basic Clin Pharmacol Toxicol*. 2005 Nov; 97(5):306-10.
- IV. Deteriorated function of cutaneous microcirculation in chronic congestive heart failure. **Edvinsson ML**, Uddman E, Andersson SE. *J Geriatr Cardiol*. 2011 Jun; 8(2):82-7.
- V. Brain natriuretic peptide is a potent vasodilator in human microcirculation but the response is down regulated in heart failure patients. **Edvinsson ML**, Uddman E, Edvinsson L, Andersson S.E Manuscript accepted.



# Summary in Swedish

## Populärvetenskaplig sammanfattning

### Vad är hjärtsvikt?

Hjärtsvikt är ett tillstånd där hjärtats pumpförmåga är nedsatt. Det betyder att hjärtat inte orkar pumpa runt den mängd blod som behövs för att ge kroppens olika organ tillräckligt med syre och näring. Den sänkta förmågan att pumpa blod ut i kroppen kan med tiden kan leda till att vätska samlas i lungorna och andra delar av kroppen.

Orsak till den nedsatta pumpförmågan kan t ex vara hjärtinfarkt, kärlkramp, högt blodtryck, rytmrubbning, framför allt förmaksflimmer, klaffel, hjärtmuskelinflammation och hög alkoholkonsumtion.

Omkring 200 000 personer i Sverige beräknas leva med symtom på hjärtsvikt. Antalet drabbade stiger med åldern och ca tio procent av befolkningen över 80 år har troligen hjärtsvikt, vilket gör det till en folksjukdom och är den vanligaste orsaken till inläggning på sjukhus.

Hjärtsvikt är ett allvarligt tillstånd som oftast kräver livslång behandling och de bakomliggande orsakerna som t.ex. hjärtinfarkt med kvarvarande kardio-vaskulära symtom som kärlkramp (angina pectoris) kan göra sjukdomen svårbehandlad. Prognosen beror på hjärtsviktens grad, ålder och andra sjukdomar. I en svensk populationsstudie av över 150 000 patienter vårdade för hjärtsvikt sjönk dödligheten mellan 5 och 10 procent per år mellan 1998 och 2000 (Schaufelberger et al. 2004). Även om prognosen vid hjärtsvikt verkar ha förbättrats är dödligheten dock fortfarande hög. Visa sviktparametrar är förenade med dålig prognos såsom kraftigt nedsatt hjärtfunktion och höga halter av olika neurohormoner (BNP) i blodet. Hälften av patienterna med hjärtsvikt dör i en pumpsvikt (vanligast vid måttlig-svår grad av hjärtsvikt) och hälften dör en plötslig död (vanligast vid lätt-måttlig grad av hjärtsvikt), sannolikt orsakad av såväl allvarliga rytmrubbningar i hjärtmuskeln som elektromekanisk dissociation med åtföljande hjärtstopp. Tidig och korrekt diagnos, optimal behandling och god omvårdnad är väsentlig för att förbättra prognosen (Dahlstrom 2004). Hjärtats uppbyggnad och pumpförmåga

Hjärtats uppgift är att ta emot blod från kroppen och pumpa det vidare till lungorna så att det kan syresättas. Sedan pumpas blodet tillbaka genom hjärtat till kroppens

olika vävnader och organ så att de får syre och näring. När man anstränger sig, går i trappor, måste hjärtat öka sin pumpförmåga. I vila pumpas ca 5 liter blod runt per minut. Vid kraftig ansträngning ökas efterfrågan på pumpförmågan till ungefär 20-25 liter per minut.

### **Vad händer när hjärtat inte pumpar som det ska?**

Det finns två huvudorsaker till att hjärtmuskelns pumpförmåga blir nedsatt vid hjärtsvikt. Den ena är när hjärtat har problem med att pumpa ut blodet (systole) på grund av minskad kraft i hjärtats muskelvägg (hjärtat har "svårt att tömma sig"). Den andra är när hjärtat har problem med att ta emot blod, under avslappningsfasen (diastole) på grund av stelhet i hjärtats muskelvägg (hjärtat har "svårt att fylla sig"). När hjärtats pumpförmåga minskar, kompenserar kroppen genom att öka pulsen för att pumpa mer blod. När hjärtat inte orkar pumpa ut blodet i samma takt som det fylls på, utvidgas hjärtat för att få plats med den ökade blodmängden. Detta leder till hjärtmuskelförtjockning, då hjärtat får arbeta hårdare och hjärtmuskeln blir större och tjockare pga. ökat arbete, mot tryck och ökad volym. Detta undersöker man med ultraljud (ekokardiografi) och EKG (Andersson, 2002).

### **Blodprov**

När en viss av tänjning av hjärtmuskelcellernas signalering av neurohormoner och en kritisk gräns uppnåtts kan inte hjärtat kompensera hjärtsvikten längre. Pulsen och hjärtstorleken kan inte öka hur mycket som helst, allt mer vätska samlas i kroppen, särskilt runt anklarna som ödem och den symtomgivande hjärtsvikten är ett faktum. Detta kallas för inkompensation. En av kompensationsmekanismerna är via neurohormonell reglering. När hjärtmuskelcellerna i förmaksväggen tänjs ut "tolkar" hjärtat det som om blodvolymen är för stor och då frisätts flera ämnen som leder till att hjärtats eget "diuretikum" signalerar via njurarna att öka diuresen. För en komplett hjärtsviktsdiagnos tas ett blodprov för att analysera koncentrationen av ett hjärtsviktsspecifikt hormon, BNP (brain natriuretic peptide) alternativt NT-proBNP. Provet ger upplysning om hjärtats fyllnadstryck, dvs. graden av belastning och kan användas för att följa sjukdomsförloppet.

### **Blodcirkulationen**

Blodcirkulationen är kroppens transportsystem. Blodet rinner i ett slutet system som bildas av blodkärlen. Blodcirkulationen bildar tillsammans med hjärtat två kretslopp. Det stora kretsloppet går från vänsterhjärtat ut till kroppen och tillbaka till högerhjärtat. Det lilla kretsloppet går från högerhjärtat till lungorna och tillbaka till vänsterhjärtat.

I våra studier har vi undersökt mikrocirkulationen i det stora kretsloppet och funnet att kronisk hjärtsvikt är förbundet med en kärl dysfunktion som innebär en försämrad förmåga för blodkärlen att vidga sig och att denna förändring är korrelerad till den låggradiga inflammation (Andersson et al., 2003) som är en del av hjärtsviktssyndromet.

## Subkutana blodkärl

De små blodkärlen i huden är lättillgängliga för mekaniska studier och därför lämpade för forskning kring kärlfunktionen hos svårt sjuka patienter med hjärtsvikt. Mätningarna i dessa kärl görs med laser Doppler teknik och innebär inget obehag för patienterna. Ett bra exempel är hur kärlens svar på lokal värme minskar med stigande ålder. De kutana (i huden) blodkärlen är av resistenstyp (motståndskärl), har stor betydelse för blodtrycksreglering och för värmeregleringen, vilket är en specialiserad funktion som däremot inte drabbas av ateroskleros.

## Forskningen har visat

Den ökade prevalensen av kronisk hjärtsvikt utgör en enorm utmaning för sjukvården. Trots att vi idag har effektiva medicinska behandlingar vid hjärtsvikt och bra läkemedel så är sjukligheten och dödligheten fortfarande betydande. Hjärtsvikt leder till en förändrad kärlreaktivitet generellt i cirkulationen, dock är betydelsen av denna endast känd i mindre omfattning.

Studierna har visat att blodkärlen på patienter med olika grad av hjärtsvikt har en generell kärl dysfunktion.

## Huvudfynden i avhandlingen är

1. Patienter med högt NT-proBNP (>5000) har en 50 % dödlighet inom 3 månader trots "state - of- the- art" behandling. Mycket återstår att göra för denna patient grupp.
2. Hudens mikrocirkulation är reducerad hos hjärtsviktpatienter beroende på blodkärlsdysfunktion, ålder och grad av inflammation.
3. En något förbättrad kärlfunktion sågs efter behandling med inflammationsdämpande läkemedel i form av B-vitamin.
4. Grad av hjärtsvikt påverkade ej sänkningen av kärlreaktiviteten.
5. BNP hade dålig effekt som blodkärlsvidgare hos hjärtsviktpatienter troligen beroende på mättnad av receptorer i blodkärl eller reduktion av G-protein signalering.



# Abbreviations

|        |   |
|--------|---|
| ACh    | acetylcholine                             |
| ANP    | atrial natriuretic peptide                |
| BNP    | brain natriuretic peptide                 |
| cGMP   | cyclic granulate mono phosphate           |
| CHF    | congestive heart failure                  |
| CNP    | C natriuretic peptide                     |
| CRP    | C-reactive peptide                        |
| DC     | direct current                            |
| ECG    | electrocardiogram                         |
| EF     | ejection fraction                         |
| ESC    | European Society of Cardiology            |
| ET-1   | endothelin-1                              |
| Hcy    | homocysteine                              |
| HF     | heart failure                             |
| IL     | interleukin                               |
| LDF    | laser Doppler flow                        |
| LDFM   | laser Doppler flow monitoring             |
| L-NAME | L-N-arginine-methyl-ester                 |
| mA     | milli ampere                              |
| NA     | noradrenaline                             |
| NO     | nitric oxide                              |
| NYHA   | New York Heart Association classification |
| PU     | perfusion units                           |
| RAAS   | renin-angiotensin-aldosterone-system      |

|              |                                 |
|--------------|---------------------------------|
| RBC          | red blood cells                 |
| sIL          | soluble interleukin             |
| SKL          | Sveriges kommuner och landsting |
| SNP          | sodium nitroprusside            |
| TNF $\alpha$ | tumor necrosis factor alpha     |

# Introduction

Heart failure (HF) is a complex syndrome with high morbidity and mortality, and a poor prognosis. Because it is a clinical syndrome definitions are imprecise; most often it is characterized by typical symptoms and objective evidence of abnormal ventricular function. The patient's condition is often assessed based on a clinical examination and the New York Heart Association functional class of disease severity (NYHA Class), an electrocardiogram (ECG), chest x-ray, Doppler echocardiography in accordance with ESC guidelines (Dickstein et al. 2008) and plasma concentrations of natriuretic peptides (O'Donoghue and Januzzi 2005).

## NYHA Classification – The stages of Heart Failure

In order to diagnose and to determine the best course of therapy physicians often assess the stage of heart failure according to the **New York Heart Association (NYHA)** functional classification system. This system relates symptoms to everyday activities and the patient's quality of life.

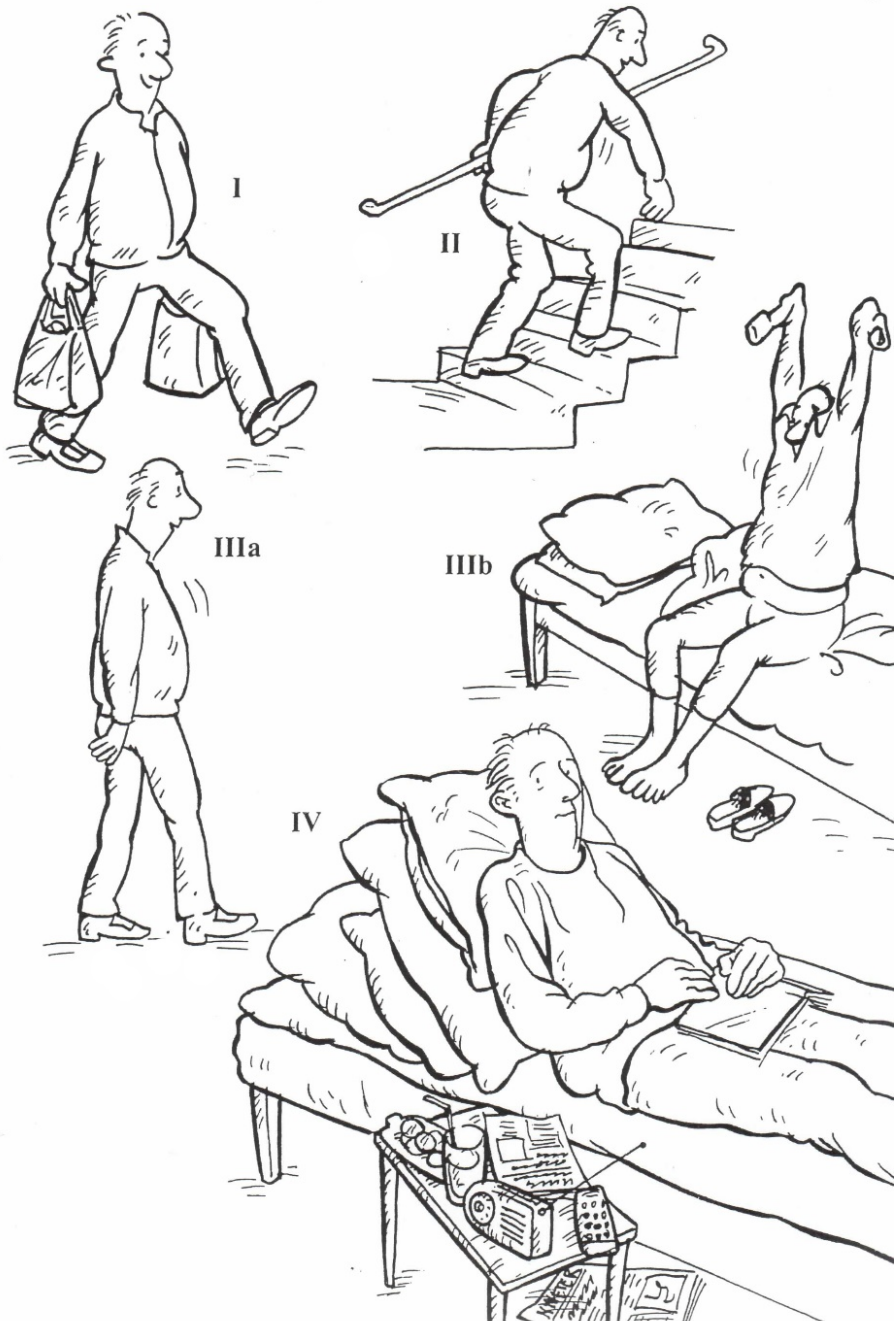
This association was formed when a group of cardiologists met in New York back in 1925 with the aim to make a uniform definition of the symptoms in the different stages of heart failure syndrome. The leading cardiologist was, Harold Ensign Bennet Pardee (1886-1973). He was appointed to the American Heart Association Committee on Research, which was charged with developing a nomenclature for standardization of diagnosis and promotion of clinical investigation in cardiac disease. Pardee edited the first four editions of *The Nomenclature and Criteria for the Diagnoses of Diseases of Heart and Blood Vessels* first published in 1928 (Pardee 1928) which is considered the origin of today's classification.

The NYHA classification was the result of that meeting and is still widely used in the everyday clinic as an advisory instrument for treatment of heart failure patients.

**Table 1**

| Class                       | Patients Symptoms   |
|-----------------------------|---|
| <b>Class I (Mild)</b>       | No limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnea (shortness of breath).                              |
| <b>Class II (Mild)</b>      | Slight limitation of physical activity. Comfortable at rest. But ordinary physical activity results in fatigue, palpitation, or dyspnea.                                  |
| <b>Class III (Moderate)</b> | Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity causes fatigue, palpitation, or dyspnea.                                     |
| <b>Class IV (Severe)</b>    | Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased. |

From: Eur Heart Failure , guidelines



**Figure 1**  
NYHA Classification – The Stages of Heart Failure. This system relates symptoms to everyday activities and the patient’s quality of life. Image with permission from Hässle Läkemedel AB.

# Pathophysiology

Heart failure (HF) is often associated with a structural abnormality of the heart. The initial injury might be sudden and obvious (e.g. myocardial infarction) or longstanding (hypertension) and in some instances, such as when the cause is not known, idiopathic dilated cardiomyopathy. Once the injury happens, subsequent maladaptive mechanisms due to pump failure lead to fluid retention and to (1) an edematous disorder, whereby abnormalities in renal hemodynamic and excretory capacity lead to salt and water retention; (2) a hemodynamic disorder, characterized by peripheral vasoconstriction and reduced cardiac output; (3) a neurohormonal disorder, pre-dominated by activation of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system; (4) an inflammatory syndrome, associated with increased local and circulating proinflammatory cytokines and (5) myocardial longstanding injury followed by pathological ventricular remodeling and the development of heart failure, which generally progresses, from risk factors to end-stage or refractory disease.

Compensatory mechanisms that are activated in HF contribute to the symptoms, signs and poor natural outcome of HF. In particular, an increase in wall stress along with neurohormonal activation leads to ventricular remodeling and this process is closely linked to the progression of HF. Management of chronic heart failure targets these mechanisms and, in some instances, results in reverse remodeling of the failing heart (Hunt et al. 2005).

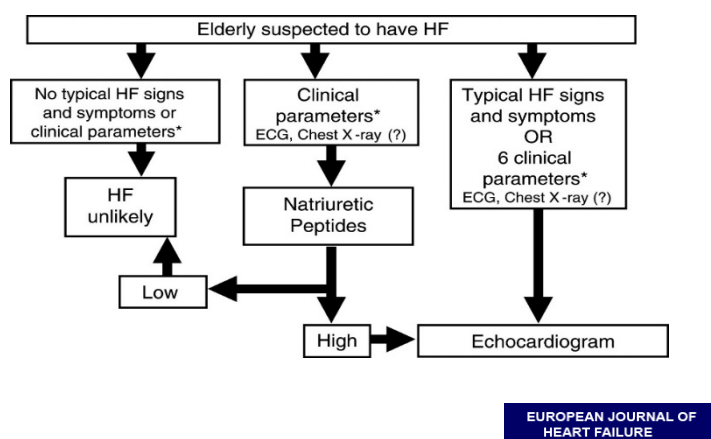


Figure 2 Potential algorithm for the diagnosis of heart failure in the elderly with increase in age, loss of appetite, absence of wheezing, low body mass index and nocturnal dyspnoea. (Parissis et al. 2002).

Prevalence of heart failure rises steeply with increasing decades of life. Estimates of heart failure incidence vary greatly between countries because it is a clinical syndrome and hence there is non-uniformity in the definition. However it is a common finding that the prevalence of HF increases with age. In a London study the incidence was 0.2 in the age group of 45-55 years and 12.4 per 1000 person-years (Cowie et al. 1999). In individuals 85 years or older, the incidence of HF was 44 per 1000 person-years in a study from Rotterdam that evaluated symptoms and relevant drug use (Mosterd et al. 1999). From the Framingham Heart Study we learned that lifetime risk of developing heart failure after the age of 40 years is nearly 20% in both men and women (Lloyd-Jones et al. 2002). Community-based assessments show that the affected individuals are most likely old, female and have associated comorbidity. This is in agreement with the incidence of HF in the Swedish population and from our National Board of Health and Welfare and Diagnosis Register (Socialstyrelsen, SKL): The report contains about 200,000 individuals with symptoms of HF and shows about the same number with fewer symptoms, e.g. HF with preserved ejection fraction. The mean age of patients diagnosed with HF is, in this report, 75 years. It is a chronic disease with one year mortality of about 20% (Edner and Lund, 2013). Every year 20,000 - 30,000 new patients are diagnosed with HF in Sweden (Swedberg et al., 2005). The comorbid factors include most of the well-known cardiovascular risk factors.

Extensive ischemic heart disease results in pump failure, and its role in HF is obvious. However even small infarcts may modify contractility and induce arrhythmia. The role of diabetes mellitus is often overlooked, it has effects on metabolism in general and strongly affects vascular function, (Kumar and Clark, 2005). Systemic hypertension is the most frequent and well-described comorbidity, relevant to both systolic HF and HF with preserved ejection fraction. The contribution of hypertension as an underlying cause of heart failure has been and is still clearly underestimated. Perhaps it is because this diagnosis is usually embedded within ischemic disorders and in other causes.

## BNP and NT-pro BNP

It is more than 3 decades since de Bold (1981) first presented evidence for the role of the heart as an endocrine organ that secretes natriuretic peptide hormones. This finding led to the definition of the natriuretic family of peptides, consisting of three structurally related peptides, that contributes to homeostasis in the circulation. Atrial natriuretic peptide (ANP) is mainly produced by the cardiac atria, and B-type natriuretic peptide (BNP) is primarily released by the cardiac ventricles. C-type natriuretic peptide (CNP) is mainly found in the human brain and in vascular endothelial cells, acting as a paracrine hormone with low circulating levels (Ruskoaho,

1992; Melo et al., 1999). The responses are mediated by three subtypes of natriuretic peptide (NP) receptors. The natriuretic peptide receptor type A (NPR-A) and type B (NPR-B) are guanylyl-cyclase coupled receptors (Koller et al., 1992). The third natriuretic peptide receptor (NPR-C) is considered as clearance receptor for natriuretic peptides. Both ANP and BNP can stimulate NPR-A and NPR-B, but with some potency variations.

The physiological role of ANP and BNP in the circulation can be regarded as that of “functional” antagonists to the renin-angiotensin system. The principal actions of ANP and BNP are reduction of peripheral vascular resistance, hypotension, natriuresis and diuresis. It is well known that both ANP and BNP are elevated in HF and that the degree of elevation correlates with severity of HF. The literature on natriuretic peptides is vast, and for this reason the present thesis has focused on BNP and the more stable clinical marker NT-proBNP, which is N-terminal inactive fragment of the circulating prohormone.

### **B-type natriuretic peptide (BNP)**

BNP is a 32-amino-acid polypeptide produced by the atrial and ventricular cardiomyocytes. It was first identified in the porcine brain, and is often referred to as brain natriuretic peptide. NT-proBNP has 76 amino acids and has a longer half-life and is more stable in the circulation. Thus it is the marker that is most commonly measured in hospital laboratories. The concentration of BNP appears to be higher in atrial than in ventricular tissue. Secretion of BNP is regulated by cardiomyocyte wall tension and is proportional to the degree of stretch. Any cause of functional volume overload will cause increased BNP production which contributes to cardiac failure. Other factors that can result in volume overload are atrial fibrillation, hypertension and valve disease, but to a much less extent. In addition to ventricular wall stress, cardiomyocytes can also be stimulated to release BNP by other factors such as norepinephrine, endothelin-1, proinflammatory cytokines and ischemia (Magga et al., 1994; Cameron et al., 2000; Bianciotti and de Bold, 2001). The studies on B-type natriuretic peptide measurement have been entirely consistent in demonstrating diagnostic and prognostic value across a range of clinical scenarios. NT-proBNP is a very robust prognostic marker at all stages of chronic heart failure and for all related clinical outcomes.

Chronic elevation of natriuretic peptides has been reported to result in hyporesponsiveness to ANP (Leitman et al., 1986; Koeller and Osborn, 1991; Imura et al., 1992; Drewett et al., 1995), but the extent is debated. Komarek et al., (2004) showed in healthy volunteers that intra-arterial ANP into the forearm resulted in vasodilation. This response was unchanged after short-term infusion of ANP but reduced in the group of subjects that received long-term infusion. Thus this is evidence for rapid desensitization of the guanylyl-cyclase type of NPR-A in humans (Komarek et al., 2004). In our study we asked if chronically elevated natriuretic peptides in HF patients may affect the vascular responses to BNP.

# Microcirculation

Generalized microvascular dysfunction plays a role in pathophysiology of the peripheral cutaneous circulation. However it is not exactly clear how it contributes to initiation and progression of disease. The dysfunction may be a consequence of the disease; or is it may be causative. Indeed, patients with impaired coronary microvascular function also have poor regulation of the generalized micro-vasculature (Sax et al., 1987; Bondesson et al., 2011). In hypertension, the resistance vessels, arteries and arterioles, show structural changes with wall thickening. The role of cutaneous microcirculation has been carefully studied and endothelium involvement has been thought to be an underlying cause; however at present it is not thought to be the main factor in essential hypertension (Lindstedt et al., 2006).

The endothelium has a central role in regulation of vasomotor tone. In patients with HF, endothelium-dependent vasodilatation in peripheral blood vessels is impaired (Kumar and Clark, 2005), and this may be one factor of exercise limitation. The changes in endothelial regulation may be due to abnormal release of both the dilator nitric oxide (reduced) and vasoconstrictor substances such as endothelin-1 (ET-1, also formed in the endothelial cells). The activity of NO is blunted in HF while the plasma concentration of ET-1 is elevated in patients with HF (Valdemarsson et al., 1994; Wackenfors et al., 2004). The ET-1 level correlates with the degree of HF.

Evidential findings have been reported of correlation between abnormalities of cutaneous and retinal microvasculature in diabetic patients (Chang et al., 1997). Today the cutaneous model is widely used as a clinically accessible microcirculatory bed for the study of vascular mechanisms in many diseases, such as hypertension, diabetes and other cardiovascular risk factors (Lindstedt et al., 2006; Levy et al., 2008; Feihl et al., 2006). The issue of how representative the microcirculation is in the skin and how it reflects on inner organs and disease is yet to be investigated. Therefore as a novel understanding of chronic heart failure, we have used a technique to study human cutaneous microcirculation as a surrogate marker of systemic microvascular function in a complex disease such as CHF. One of our aims was to determine how well responses in the cutaneous microcirculation reflect the stage of HF disease.

There exist many methods to study different parts of the human circulation and at different levels. Our choice was to use an atraumatic and clinically useful method that could be applied to patients with severe disease such as end stage HF. A laser Doppler probe was used, to measure movements of blood cells and flow, in combination with

local iontophoresis of vasoactive substance such as acetylcholine (endothelium-dependent dilator) and sodium nitroprusside (vascular smooth muscle dilator).

# Aims

The general aim of the work presented in this thesis was to investigate the peripheral microcirculation in congestive heart failure patients and more precisely the role of BNP.

The specific aims were:

- I. To assess the long-term prognostic information provided by a single measurement of NT-proBNP in elderly CHF patients admitted to hospital,
- II. To investigate whether changes in vascular reactivity in CHF patients can be detected in cutaneous microvessels and if these changes are due to endothelial dysfunction, age and/or inflammation,
- III. To examine if supplementation with vitamins B<sub>6</sub>, B<sub>12</sub>, and folate could normalize the hyperhomocysteinaemia in CHF patients and improve the cutaneous vascular reactivity,
- IV. To test the hypothesis that dysfunction in vascular function correlates with the severity of CHF and aging and determine whether these two influences have a synergistic effect on the microvasculature,
- V. To investigate if the high levels of circulating BNP in CHF patients affect the response of microvascular natriuretic receptors.



# Methods

## Patients

**Paper I** was conducted in CHF patients older than 65 years in whom NT-proBNP was measured and who sought medical attention at the emergency department at Lund University Hospital during 2003-2004. To reach high specificity we set a NT-proBNP level of 2000 pg/ml or higher as an inclusion criterion. In this study 184 men and 181 women met this criterion. All heart failure patients were thus treated according to clinical routine and were followed up in the general population register for survival during the two years after inclusion.

In **paper II**, 15 CHF patients were compared to a control group of healthy age- and sex-matched controls. In order to study the influence of age on vascular reactivity, six healthy young adults were also studied. Several different markers for inflammation and pro thrombotic factors were measured.

In **paper III**, Fourteen CHF patients with high homocystein levels were treated with supplementation of vitamins B<sub>6</sub>, B<sub>12</sub> and folic acid for a period of six weeks. The vasodilatory responses to ACh and SNP were measured in the cutaneous microcirculation before and after intervention.

In **paper IV** the study population consisted of three groups: 20 severely ill, hospitalized CHF patients with NYHA class IV, mean age 85.5 years, were compared to a group of 15 CHF patients obtained from the out patients clinic, mean age of 76.5 years. They were considered clinically stable with NYHA II and further compared to 10 healthy controls, mean age of 67.6 years with no clinical signs of HF.

In **paper V** 15 patients with CHF (mean age of 77.8 years), with NYHA class III/IV symptoms were compared with 10 healthy age- and sex-matched subjects recruited from the community registry with a mean age of 78.8 years.

# Ethics

All investigations conformed to the principles outlined in the Declaration of Helsinki, Seoul 2008. The Ethics Committee of Lund University approved of the protocol. Written informed consent was obtained from all patients and healthy controls by the investigators before they were entered into the study and this was verified in the electronic medical charts.

## Iontophoresis method

### Introduction

The method of iontophoresis was first described by Pivati in 1747 (Pivati, 1747). Galvani and Volta, two well-known scientists working in the 18<sup>th</sup> century, combined the knowledge that electricity can move different metal ions, and the movements of ions produce electricity. The method of administering pharmacological drugs by iontophoresis became popular at the beginning of the 20<sup>th</sup> century due to the work of Leduce in 1900 who introduced the term *iontotherapy* and formulated the laws for this process (Leduce, 1900).

Iontophoresis is defined as the introduction, by means of a direct electrical current, of ions of soluble salts into the tissues of the body for therapeutic purposes (Singh and Maibach, 1994). It is a technique used to enhance the absorption of drugs across biological tissues, such as the skin. Another method for drug delivery through the skin, called phonophoresis, uses ultrasound instead of an electric current. In clinical practice, iontophoresis devices are used primarily for the treatment of inflammatory conditions in skin, muscles, tendons and joints, such as in temporo-mandibular joint dysfunctions. More recently, iontophoresis is used in combination with laser Doppler technology as a diagnostic tool in diseases compromising the cutaneous vascular bed.

### Principle of iontophoresis

By definition, iontophoresis is the increased movement of ions in an applied electric field. Iontophoresis is based on the general principle that *like* charges repels each other, and *unlike* charges attract each other. An external energy source can be used to increase the rate of penetration of drugs through the membrane. When a negatively charged drug is to be delivered across an epithelial barrier, it is placed under the negatively charged delivery electrode (cathode) from which it is repelled, to be

attracted towards the positive electrode placed elsewhere on the body. In anodal iontophoresis (positively charged ions), the electrode orientation is reversed.

The choice of drug is of importance, depending on whether the compound is unionized or ionized. Non-ionized compounds are generally better absorbed through the skin than ionized substances. The penetration of ions across the skin or other epithelial surfaces is usually slow due to excellent barrier properties. Many drug candidates for local applications only exist in an ionized form, which makes effective membrane penetration impossible.



**Figure 3**

The drug delivery probe (Periflux system 383) which contains the drug that is positively charged (eg. acetylcholine).

### **Factors affecting iontophoretic transport**

Many factors have been shown to affect the results of iontophoresis. These include the physio-chemical properties of the compound (molecular size, charge, concentration), drug formulation (type of vehicle, buffer, pH, viscosity, presence of other ions), equipment used (available current range, constant vs. pulsed current, type of electrode), biological variations (skin site, regional blood flow, age, sex), skin temperature and duration of iontophoresis.

Iontophoresis has mainly been used for therapeutic purposes. However, in combination with the laser Doppler technique; it is possible to use this delivery mode to study the influence of drugs on the vascular bed. Until now, the combination of LDFM (Laser Doppler Perfusion Monitoring) and iontophoresis has been used mostly as a diagnostic tool for diseases affecting macro- and microcirculation and the controlling regulatory nerves. When using iontophoresis as a diagnostic instrument, the following factors have to be considered.

### *Influence of pH*

The pH is of importance for the iontophoresis delivery of drugs. Optimum delivery is seen with a compound that exists predominantly in an ionized form. Since hydronium ions are small, they can penetrate the skin more easily than larger drug ions. It is also important to keep the pH as close as possible to 7, particularly when working with vasodilators. When the pH decreases, the concentration of hydrogen ions increases and a vascular reaction (vasodilation) is initiated because of C-fiber activation. At pH 5.5 and below, there is an increased risk for vascular reactions due to the high concentration of hydrogen ions rather than the compound used.

### *Current Strength*

There is a linear relationship between the observed flux of the number of compounds and the applied current. With the electrode area of 1 cm<sup>2</sup> (PF383) used in Papers II-IV, the current is limited to 1 mA due to patient comfort considerations. The current should not be applied for more than three minutes because of local skin irritation and burns. With increasing current, the risk of non-specific vascular reaction (vasodilatation) increases. Such a vascular reaction is initiated after a few seconds of iontophoresis with de-ionized water at a current of 0.4 to 0.5 mA /cm<sup>2</sup>. This latter effect is probably due to the current density being high enough within a small area to stimulate the sensory nerve endings, causing reactions such as the release of sensory signal substances such as CGRP (calcitonin gene-related peptide) and substance P from C-fiber terminals.

### *Ionic Competition*

In a solution of sodium chloride, there is an equal quantity of negative (Cl<sup>-</sup>) and positive (Na<sup>+</sup>) ions. Migration of a sodium ion requires that an ion of the opposite charge is in close vicinity. The latter ion of opposite charge is referred to as a counter-ion. An ion of equal charge but of a different type is referred to as a co-ion. When using iontophoresis, it is important to know that pH adjustment is performed by adding buffering agents. The use of buffering agents adds co-ions which are usually smaller and more mobile than the ion to be delivered. This results in a reduction of the number of drug ions to be delivered through the tissue barrier by the applied current.

### *Drug Concentration*

Depending on the drug used, the steady-state flux (ion movement) has been shown to increase with increasing concentration of the solute in the donor compartment, i.e. in the delivery electrode. A limiting factor to be considered is the strength of the current used. At higher drug concentrations the transport may become independent of concentration, probably because of the saturation of the boundary layer relative to the donor bulk solution (Phipps et al., 1989).

### *Molecular Size*

It has been shown that the permeability coefficient decreases, with increased molecular size (Yoshida et al., 1993). However, there are certain solutes with relatively high molecular size (e.g. insulin, vasopressin and several growth hormones), which have also been shown to penetrate the skin barrier into the systemic circulation.

### *Current- or Continuous vs. Pulsed mode*

Application of a continuous current over a long period of time can modulate iontophoretic delivery. Continuous direct current (DC) may result in skin polarization, which can reduce the efficiency of iontophoresis delivery in proportion to the length of current application. This polarization can be overcome by using pulsed DC, a direct current that is delivered periodically. During the “off time” the skin becomes depolarized and returns to its initial unpolarized status. The enhanced skin depolarization using pulsed DC can, however, decrease the efficiency of pulsed transport if the frequency is too high (Burnette and Bagniefski, 1988). Enhanced iontophoretic transport has been reported for peptides and proteins by using pulsed DC compared to conventional DC (Chien and Banga, 1989). Most of the drug ions used for diagnostic purposes in combination with iontophoresis and LDPM are small in size and, the time needed for an effect is relatively short (5-120 sec), compared to when iontophoresis is used for therapeutic purposes (20-40 min).

### *Physiological Factors*

Iontophoresis reduces intra- and inter- subject variability in the delivery rate. This is an inherent disadvantage with the passive absorption technique. Experiments *in vivo* and *in vitro* give support for clinical findings that there are small differences in the flux rate following transdermal iontophoresis between males and females, as well as between hairy and hairless skin. The status of the vascular bed is also important; for instance, a pre-constricted vascular bed decreases the drug flux through the skin while a dilated vascular bed increases the yield of the drug through the skin.

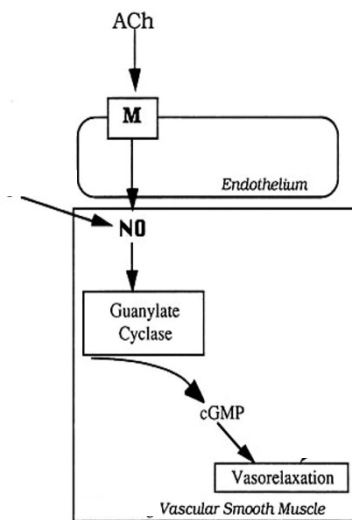
### *Optimizing Iontophoretic Transport*

Iontophoretic transport can be regulated by varying the applied current density and area of application. A current density that is too high may be unpleasant for the patient. It has been recommended not to use current set that result in more than 500 mA/ cm<sup>2</sup>. This should be compared with our use of 0.2 mA for acetylcholine and 0.1 mA for sodium nitroprusside, as common test substances (Papers 2-5). At high current densities, there is a significant risk for unspecific electrically- mediated vasodilation that is not drug related.

The pH of the formulation should be optimized to ensure maximum ionization of the compound. To prevent pH drifts during the iontophoresis, the choice of

electrodes is important. With correct electrode material, decreased solubility and precipitation of the compound are avoided.

The skin area should be cleaned before iontophoresis is carried out with deionized water or 70 per cent alcohol. Cleaning will decrease the current needed and minimize the risk for local spots of high current density, which could result in C –fiber activation, vasodilatation and local micro-burns.



**Figure 4**

Sodium nitroprusside acts like an NO doner, which then acts via cGMP to dilate the vasular smooth muscle.

### *Disadvantages of Iontophoresis*

Major side-effects are very rare when using iontophoresis as a diagnostic tool. However minor reactions such as itching, erythema and general irritation of the iontophoretic skin surface are common. There is an increased risk of minor reactions if the time exposure or current are increased for some drugs like histamine, capsaicin and acetylcholine. Some drugs induce long-lasting skin pigmentation after iontophoretic application, where the intensity of skin discoloration is proportional to the exposure time.

The current density across the pores in the skin may be higher than the current per unit area applied, depending on the density of pores in a given area. These spots of high current density increase the possibility of current-induced skin damage.

Burnette and Ongpipattanakul (1988) showed that the skin resistance was always less than the initial value when a current of *0.16 mA was applied for 10 minutes*. This may result in permanent skin damage. This phenomenon may explain the sudden vascular response with iontophoresis of deionized water, which seems not to be related to dose. Under the microscope, small spots of skin damage within the pore area could be recognized. The vasodilatation initiated in this way may be caused by activation of nociceptive fibers terminating in the epidermis, which initiate an axon reflex mediated vascular response.

### *Contraindications*

Contraindications for iontophoresis are important in patients with higher susceptibility to applied currents. Previously patients carrying pacemakers or implanted devices were considered not suitable for this technique, but this is no longer a problem due to modern standards. Patients that are hypersensitive to the drugs used, or if they have broken or damaged skin surfaces are not suitable to test and should not be investigated with this technique (Andersson et al., 2003).

## The Laser Doppler Method

Light is transmitted to the tissue via a fiber-optic probe. When this light hits moving blood cells, it undergoes a change in wavelength (Doppler shift). The magnitude and frequency distribution of these changes are directly related to the number and velocity of blood cells, i.e. the blood perfusion. Measurements are expressed in arbitrary Perfusion Units (PU). Full linear correlation to absolute perfusion value is achieved using Premed's innovative analysis technology (including a linearization function to avoid underestimation in highly perfused tissues) and calibration using automatic instrument zeroing and Premed's Motility Standard (Bollinger et al., 1991).

### **Percent Change Report**

At baseline blood flow the vasomotion of the microvessel is followed until conditions have stabilized. After the stimulation, the measurement is recorded when vasodilation reaches the peak. This is then followed by comparison of the mean value from one testing phase to another. Perfusion Units are determined by the use of calculating the percentage of changes after stimulation.

## Heat analysis and maximum vasodilation report

The cutaneous vascular bed serves as an important reserve in the regulation of body temperature. However at rest, the blood flow of the skin and mesenteric circulation greatly exceeds the nutritional requirements of the vascular beds (Zelis et al., 1975). Therefore, skin and mesenteric circulation are also functional reservoirs in this situation, when increased blood supply is required elsewhere e.g. in the skeletal muscles during physical exercise, or when blood flow to vital organs is threatened, e.g. due to blood loss or cardiac failure

The perfusion change after local heating (e.g.  $+44^{\circ}\text{C}$ ) is a measure of the tissue reserve capacity. The report includes calculations of mean values before and after heating, percent change, and slope and time from heat marker to max area.

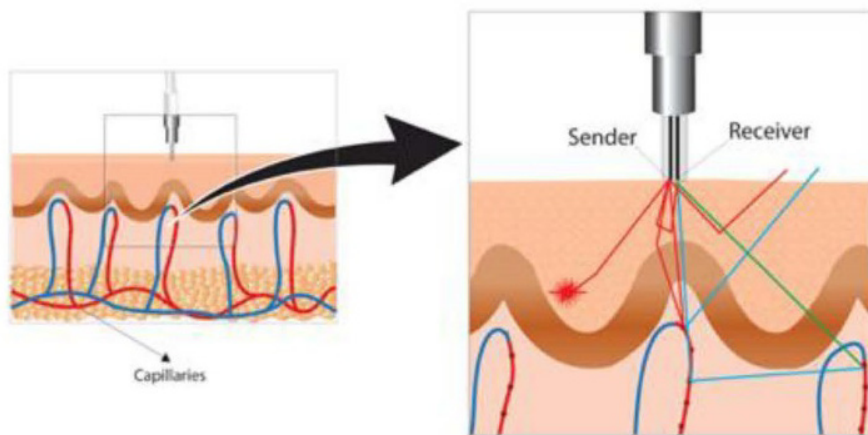


**Figure 5**

The Periflux 5000 System with the big black probe containing the substance of choice, here acetylcholine, because it is connected to the red crocodile wire (anode). The smaller probe (in the center) with a laser light is now acting as a control measurement of the blood flow. To the right we see the instrument of the battery where the current is engendered from.

## Protocol

All studies were performed in a temperature-controlled room at +22-24°C, with the subjects resting in the supine position. The skin of the lower arm was gently cleaned with an alcohol swab and the iontophoretic applicators/fiber optic probes were applied to the forearm. The basal blood flow was studied for 2 minutes after which ACh was transferred by iontophoresis (anodal current, 0.2 mA for 20 s). Current alone did not affect the resting blood flow (results not shown). Repeating the iontophoretic stimulation five times at 60 s intervals produced a stimuli-response curve. Endothelium-independent vasodilation was studied by iontophoresis of SNP as above (cathode current, 0.1 mA for 60 s). The stimulation was repeated four times at 60 s intervals. Finally, the response to heat was measured following local warming to +44°C for 10 min. (Papers II-IV). The vasodilatory effect of BNP (anodal current, 0.2 mA for 60 s) was measured as above with the stimulation repeated four times at 60 s intervals (Paper V).



**Figure 6**

The principle of the Laser Doppler technique for assessing blood flow is to measure the Doppler shift – the frequency change that light undergoes when reflected by moving objects, such as red blood cells. Measurement of the RBC motion is recorded continuously in the outer layer of the tissue. The number of red cells times their velocity is reported as microcirculatory perfusion units.

# Chemical analysis of NT-proBNP

## *Plasma-NT-proBNP*

ProBNP, a precursor form of BNP, consists of 108 amino acids and is cleaved in conjunction with release into active BNP (amino acids 77-108) and an N-terminal inactive fragment (NT-proBNP, amino acids 1-76). The physiologically active fragment BNP has a short halftime (<30 min) while the inactive N-terminal fragment has longer halftime (several hours). Thus the clinical variability of NT-proBNP is less than that of BNP in plasma. The analysis of NT-proBNP is performed with an immunometric sandwich method with Electro C Hemi Immunoassay (ECLI) detection method.

**Table 2**  
Reference intervals

|        |           |           |
|--------|-----------|-----------|
| Women: | <60 years | <150 ng/L |
|        | >60 years | <300 ng/L |
| Men:   | <60 years | <100 ng/L |
|        | >60 years | <300 ng/L |

**Table 3**  
Measurement intervals

|                         |               |
|-------------------------|---------------|
| Measurement:            | 5–35 000 ng/L |
| Functional sensitivity: | 50 ng/L       |

The analyses were made at the Department of Clinical Chemistry, Lab Medicine, Lund University Hospital, Lund University, Lund, Sweden. The method is certified. Accreditation ISO-SWEDAC-1309

# Results and Comments

## Paper I.

This paper addresses the hypothesis that an acute measurement of NT-proBNP blood levels has predictive value regarding the long-term outcome for HF patients.

During the last two decades there has been a substantial reduction in the mortality of patients with heart failure, most probably due to the introduction of drugs regulating renin-angiotensin, aldosterone and beta-receptor. Although there is less mortality, the prevalence of HF is still high; thus increasing numbers of patients are living longer with chronic HF. During the disease progression several compensatory mechanisms are in play; one of these are natriuretic peptides which show enhanced production and release in a manner that correlates intimately with the degree of HF (Motiwala and Januzzi, 2013).

BNP and NT-proBNP are the current gold-standard biomarkers for evaluations of prognosis in chronic HF. They give independent information on the risk of disease progression, ventricular remodeling and hospitalization for HF. Although a single measurement of BNP and NT-proBNP may not fully show the outcome, we currently examined the usefulness of an analysis of NT-proBNP in an emergency situation and followed the patients for up to 2 years. Many of the patients were quite elderly providing us with unique insights into chronic HF sufferers of advanced age.

The first problem encountered in this study was that there were no reference levels for the analysis of NT-proBNP for subjects older >65 years available at Clinical Chemistry (Lund). Since our patients were much older than >65 years the reference levels provided by the present work have now been implemented as an reference for this age group in the analysis of NT-proBNP at Clinical Chemistry, Lund University hospital.

In our group of patients of total 365, older than 65 years (184 men; mean age, 78+/-0.8 years/181 women; mean age, 82+/-0.6 years) seeking medical attention at the Lund University Hospital Emergency Clinic during a 2-year period and who had an NT-proBNP value >2000 pg/mL were followed up for survival. Mortality in our population was 21% after 3 months, 35% after 1 year, and 40% after 2 years. Multivariate analysis indicated that the NT-proBNP level and the New York Heart Association (NYHA) functional class were stronger predictors of mortality than were

echocardiographic estimation of left ventricular ejection fraction or chest radiography. Patients who survived the first year were younger, had higher systolic blood pressure, had lower plasma creatinine, had lower inflammatory activity, and were treated with lower doses of furosemide. The results (Paper I) indicate that in this population, NT-proBNP level together with assessment of NYHA class gives the best prognostic information of 1-year mortality (Paper I).

Our study is in agreement with other researchers that NT-proBNP levels provide important prognostic information independent of echocardiographic correlates (Groenning et al., 2004).

Already in 1994, Valdemarsson (Valdemarsson et al., 1994) described the relationship between ANP and survival in CHF in a study with a similar design as ours where a single sample of ANP was taken in a hospital setting and then the CHF patients were followed for survival. The neurohormonal influence of high levels of noradrenalin (NA) (Cohn et al., 1984) and also atrial natriuretic peptide (Gottlieb et al., 1989) have been related to higher rates of mortality. Valdemarsson found that there was a significant relationship between NA and ANP levels and survival time confirming the importance of NA and ANP as prognostic markers in CHF. The increased level of ANP seems accurately to reflect the severity of CHF. Thus the beneficial natriuretic and vasodilating functions of ANP do not seem to offset the adverse effects of sympathetic nervous activation in CHF.

In our study of 365 CHF patients, multivariate analysis did not fall out for the commonly known fact that diabetes is a cardiovascular risk factor combined with high mortality. This has also been reported by other groups. Although there are conflicting data, it seems likely that plasma NT-proBNP and BNP levels might prove useful in screening for left ventricular abnormalities in diabetic patients, in predicting the presence of silent myocardial ischemia, in providing a prognostic index in diabetic patients following acute myocardial infarction, and in predicting mortality and cardiovascular outcome in patients with diabetes (Groop and Thomas, 2005). Taken together we can conclude that NT-proBNP level together with assessment of NYHA class gives the best prognostic information of 1-year mortality.

## Paper II

In the pathophysiology of HF several mechanisms are at play:

1. Abnormality of renal hemodynamics with salt and water retention;
2. Peripheral vasoconstriction and reduced cardiac output;
3. Neurohormonal dysbalance;
4. Low grade of inflammation;
5. Myocardial remodeling (Poole-Wilson, 1988).

The dilatory response to the muscarinic receptor agonist metacholine (similar to ACh), has been reported to be blunted in patients with CHF (Kubo et al., 1991). Furthermore, Hirooka (Hirooka et al., 1994) found with pletysmography that the response to intra-arterial ACh was blunted in CHF patients as compared to control subjects. This indicates an impaired endothelial function or perhaps a change in muscarinic receptor function. In this study there was also a depressed response to SNP, which is in agreement with our findings (Paper II).

There exists relatively little information on the cutaneous microcirculation in CHF; however this vascular bed may give indication of the re-distribution of flow by the failing heart. The role of the microcirculation has been emphasized in hypertension (Feihl et al., 2006). Impaired vasomotor reactivity on microvascular vessels is thought to be a key mechanism in the pathophysiology of idiopathic hypertension and has a major contribution to CHF.

With regards to CHF little is known about vascular reactivity in these patients and our research has been designed to fill this gap. Functional alterations in microvascular control may both be an indication of CHF progress but may also provide understanding of underlying pathophysiology. Reduced endothelial-dependence and changes in vascular smooth muscle may differentially modify the circulation in CHF.

As the first issue to consider in CHF studies is “do age and inflammation matter”? To address this we investigated whether alterations in vascular reactivity can be detected in the cutaneous microvessels in CHF patients. We tested if such changes were due to endothelial dysfunction, affected by increasing age and related to an ongoing inflammation. The responses to local warming and iontophoretically administered endothelium-dependent and -independent vasodilators were investigated in healthy young adults, healthy elderly adults and elderly adults with CHF. The results were correlated with plasma concentrations of vascular risk factors and markers for endothelial dysfunction and inflammation.

The vasorelaxation responses were reduced in all elderly groups relative to younger subjects but they were attenuated further in the CHF group. The latter group also had increases in levels of several markers associated with inflammation, higher blood glucose and homocysteine levels, a lower low-density lipoprotein-cholesterol and a

rise in the concentration of von Willebrand factor, indicating a prothrombotic endothelial function. The severity of heart failure, measured as the plasma level of brain natriuretic peptide, correlated with the intensity of inflammation and with the changes in vascular risk factors and endothelial function.

We concluded that the reactivity of the cutaneous microvessels is reduced with age (Egashira et al., 1993). It was reported that endothelium-dependent vasodilation was linearly reduced with age in human coronary arteries. We also found the presence of CHF causes a further impairment in vascular responses. There is endothelial dysfunction in CHF, but it is uncertain to what extent this contributes to reduced vasodilatory capacity. The inflammatory response appears central for many of the manifestations of the CHF syndrome.

## Paper III

The CHF condition is repeatedly found to be associated with a low grade of inflammation with elevated CRP, IL-6, sIL-2r and uric acid (Andersson et al., 2003). Others have reported increases in TNF-alpha and IL-8 (Gullestad et al., 2001). It is possible that homocysteine is elevated as a part of an acute phase response as described both after stroke (Lee et al., 2010) and myocardial infarction (Schnyder et al., 2002). It was also noted that one third of our patients from earlier study populations had a plasma homocysteine of  $> 12 \mu\text{M}$ , which was higher than in healthy controls (De Vriese et al., 2002). Because there were signs of low grade inflammation in CHF, we designed a study to examine this issue to some degree (Paper III). We examined if supplementation with the vitamins B<sub>6</sub>, B<sub>12</sub> and folate could normalize the enhanced levels of homocysteine and if so improve the associated clinical parameters. This was an open study without placebo control on CHF patients with plasma homocysteine  $> 15 \mu\text{M}$ . Measurements of cutaneous vascular reactivity, blood pressure, inflammatory activity and endothelial function were performed before and after intervention, with intra-individual comparisons. The treatment reduced homocysteine to near normal values and enhanced the hyperemic response to acetylcholine related to the response to heat. The mean arterial blood pressure and pulse rate were reduced. There was no significant effect on inflammatory activity, plasma levels of von Willebrand factor, subjective health quality or the hyperemic responses to sodium nitroprusside or to local warming. The enhanced level of homocysteine in some CHF patients is multifunctional in origin. Folate deficiency, inflammatory activity and reduced renal function could be contributing. It is suggested that supplementation with B-vitamins can improve the vasodilatory capacity and reduce the blood pressure but additional studies are required to confirm this. It should be noted that in this present patient group almost no one had B<sub>12</sub> or folate deficiency.

In our results we saw that the blood pressure was significantly lower after intervention. Since this study was open and without placebo control it is not possible to conclude if this was due to the increased adherence to the prescribed pharmacological treatment or to placebo effect. There are however, previous reports that treatment with pyridoxine plus folic acid had a reducing effect on the blood pressure (van Dijk et al., 2001). A large American cohort study showed that higher folate intake is associated with reduced risk of incidence of hypertension (Forman et al., 2005). Thus it might be hypothesized that vitamin supplementation has direct blood pressure-reducing effect in CHF patients with elevated levels of homocysteine, possibly mediated by an improvement of vascular function. However this needs to be confirmed in larger and better controlled studies. Also the ACh-induced hyperemia was significantly elevated, however, as compared to the heat dilatory response. This suggests that high levels of homocysteine contribute to the impaired vasodilatory capacity in heart failure patients but it is also possible that improved compliance to prescribed medication could have affected the results.

The role of homocysteine and the relevance to the prevention and treatment of cardiovascular disease has been questioned. Meta-analysis (Khandanpour et al., 2009), and Cochrane Database Systematic Review (Hansrani and Stansby, 2002) found that there were no adequate trials of the treatment of patients with peripheral vascular disease who had elevated plasma homocysteine. A trial of the effect of folic acid and 5-methyltetrahydrofolate (an active form of folic acid) supplementation was found to improve the ankle-brachial pressure index and the pulse-wave velocity in patients with peripheral arterial disease. These measures improved with 16 weeks of treatment (Khandanpour et al., 2009).

Higher plasma homocysteine levels are associated with a higher risk of cardiovascular, cerebrovascular and peripheral disease. Little is known about heart failure but our study adds to that knowledge. It is possible that homocysteine is an inflammation marker, and thus associated with low grade inflammation in CHF. Randomized trials are needed to see if the supplementation used here improves outcome in patients with high homocysteine levels (Abraham and Cho, 2010). Our study, however limited in numbers, provides a suggestion but no proof.

## Paper IV

Based on the progressive rise in NT-proBNP with severity of CHF, and the reduction in EF, we postulated that these changes would also be reflected in the vasomotor responses to ACh, SNP and heat in CHF patients.

In the population from our Hospital, we observed that the CHF patients are older before they exhibit severe degrees of CHF. In order to evaluate if the cutaneous

microcirculation is affected by age we tested this question both in healthy aged subjects and on elderly CHF patients. This group of patients is seldom studied even though they are so frequently seeking hospital care for worsening of their CHF symptoms. We have previously shown that vascular reactivity is reduced with increasing age. In very old patients with severe CHF, vascular function is further compromised by a combination of heart failure and aging.

In the present study we aimed to investigate these phenomena by studying if age and severity of CHF could have a synergistic effect on the microvasculature; or if the vascular dysfunction mainly is an early sign in the heart failure syndrome.

Cutaneous forearm blood flow was measured in three groups: Group 1 ( $85.5 \pm 4$  years), heart failure patients with New York Heart Association class IV (NYHA IV) and with a NT-proBNP level  $\geq 5000$  ng/L; Group 2 ( $76.5 \pm 2$  years), heart failure patients with NYHA II and NT-proBNP  $\leq 2000$  ng/L, and Group 3 ( $67.6 \pm 3.0$  years), healthy controls with no clinical signs of heart failure.

All patients with heart failure had significantly reduced vascular reactivity independent of the mode of stimulation (ACh, SNP or heat) when compared to age-matched healthy controls. However, the responses did not differ between the two groups of heart failure patients. Thus cutaneous vascular reactivity was reduced in heart failure patients but it did not correlate with the severity of the condition or age of patients.

The reason behind this observation is not known but several possibilities might be advanced. As the disease progress from early stages of heart failure the circulation is designed to counterbalance with release of different hormones and signal molecules, stored in endothelium, nerve fibers and in the heart and kidney. These signals interact in turn with the circulation at multiple points in order to counterbalance. It is likely, however, that much of the peripheral counterbalancing mechanisms will have already been activated to their limit early on in HF. Another possibility is that the second messenger step is exhausted as noted in biopsies of CHF hearts for guanylyl-cyclase (Dickey et al., 2007). Clearly this field of research on counter balancing mechanisms in CHF deserves further consideration; and it might lead to better understanding and treatment via novel mechanisms.

## Paper V

ACE inhibitors, aldosterone and beta-adrenoceptor blockers all have a major influence on chronic CHF mortality rates. However, as the failing heart problem progress further, ways to alleviate the symptoms are required. One way is the use of devices such as pacing, defibrillators and, in selected cases, left-ventricular assist devices. But this is offered to only a few; hence the need for new drug therapies is

much needed. Inhibition of TNF- $\alpha$ , various aspects of the endothelin mechanisms and vasopressin have not shown superiority over conventional treatments. Currently there is a hot discussion about using BNP *per se*, as a last resort in severe CHF (Januzzi et al., 2012). Evidence is not clear to what extent this new therapy could be helpful for patients with advanced HF due to problems with hypotension. In order to elucidate this issue from our viewpoint, we designed a study to evaluate the microvascular aspects of BNP in HF patients.

BNP is normally present in low levels in the circulation but it is elevated in parallel with the degree of congestion in heart failure. The peptide mainly originates in the cardiac myocytes and is produced in large amounts in CHF. BNP has natriuretic effects just like ANP and is a potent vasodilator in the peripheral circulation (Potter et al., 2006). It is suggested that BNP could be a therapeutic alternative in severe CHF, based on its role in healthy individuals.

The clinical trials on BNP in severe CHF have not given the clinical validation that was predicted. Recently the data from >7000 patients were reviewed and infusion of BNP elicited a minor advantage at best (O'Conner et al., 2011). The reason behind this lack of effect is not known, but one interesting hypothesis has appeared (Kuhn 2003) has focused on guanylyl cyclase-A and observed that this second messenger enzyme was reduced in CHF while the natriuretic receptors were unaltered (Kuhn et al., 2004). Thus signal transduction for BNP may be impaired in the chronic HF patient.

In order to find out more about the mechanisms involved we postulated that the high level of BNP in heart failure might lead to a chronic occupation of these receptors and secondarily down regulate the natriuretic receptor vasodilator response to iontophoretic BNP. This was tested in 15 CHF patients (BNP>3000 ng/L) and 10 matched healthy controls.

We observed the expected differences in responses to ACh and to local heating between CHF and controls. Interestingly, BNP elicited a maximum dilatation of 794% change in healthy controls but only 283% change in CHF subjects ( $p<0.01$ ). Thus the response was reduced to less than half in CHF patients. Comparison of the percentage reduction by ACh and heat in HF, versus the amount of reduction in BNP responses in HF showed a difference ( $p<0.05$ ). The nitric oxide synthase inhibitor (L-NAME) totally blocked the BNP relaxation. This observation demonstrates that the BNP response in cutaneous microcirculation involves a nitric oxide dependent dilatation. The findings show for the first time that the vascular responses to BNP are reduced in CHF patients. The relaxant effect of BNP is mediated via the formation of nitric oxide. This is consistent with the hypothesis of a BNP receptor down regulation or attenuation of the guanylyl cyclase mechanism in CHF. The future studies will examine in more detail the mechanisms involved in natriuretic responses in the human cutaneous microcirculation using myography and molecular biology methods.



# Major conclusions

The classification of CHF by NYHA functional scale grade I-IV is not a precise measurement but has over the years proven to be accurately well correlated to survival. NYHA class III-IV predicts poorer chances of survival. The power of this scale is that the information of symptoms of CHF told by the patient and observed by the physician, gives a holistic notion about the prognosis and outcome of CHF. In addition to that knowledge we also get the information from the measurements of left ventricular ejection fraction. It is a well-established fact that the neurohormonal activation in CHF, such as ANP and BNP, is a very strong marker for a poor prognosis of the disease. One can assume that this neurohormonal activation is reflected in the patient's symptoms, which is not directly seen in our measurement of heart function. Therefore, it is possible for a patient to have well preserved EF, but a large increase in neurohormonal activation and with severe symptoms which foreshadow clinical worsening and the prognosis of CHF. BNP and NT-proBNP are the gold-standard biomarkers for evaluating the prognosis in CHF. The present work concludes that the NT-proBNP level together with assessment of NYHA class gives the best prognostic information of 1-year mortality in CHF.

To fill the gap of relatively little knowledge of the role of the peripheral microcirculation in CHF we introduced the technique of iontophoresis and laser Doppler flowmetry of this patient group. This non-invasive method, with no discomfort, no side effects and repeatable over time, gave us the possibility to examine the cutaneous microvascular bed in severely ill CHF patients. This group of patients could be studied in the hospital wards; they were mostly of high age, multiple illnesses, had severe CHF, often with NYHA class IV symptoms and >5000 ng/L of NT-proBNP levels, and were burdened with co-morbidity and multi-pharmacy treatments. For the first time, this technique was made available to the study of these patients. In the hospital, at bedside, we investigated this in a randomized study. Age and low grade of inflammation can affect the vascular reactivity.

It was observed that the reactivity of the cutaneous microcirculation is reduced, in part due to endothelial dysfunction and in part due to reduced smooth muscle reactivity by age; the presence of CHF causes further impairment. We also suggest that this non-invasive method of iontophoresis and laser Doppler flowmetry can be successfully used as a surrogate method to follow the status, development and prognosis of CHF in contrast to invasive methods currently used.

The research field of CHF often reports the association with low grade of inflammation. In our studies we repeatedly observed that CHF patients had elevated CRP, uric acid and cytokines (IL-6 and sIL-2r). Especially, the interleukin 2 receptor level seems to be linked to CHF. This needs to be further analysed for mechanistic interpretation.

Elevated plasma homocysteine levels are associated with high risk of cardiovascular, cerebrovascular and peripheral vascular disease. The question of elevated plasma homocysteine levels in CHF patients has not received particular attention before. We found in our study that the CHF patients benefit from B-vitamin supplements to the extent that the homocysteine levels normalized, that the blood pressure was significantly lower after intervention and that the vasodilatory response to ACh was significantly improved. This suggests that the vasodilator capacity can be improved in CHF patients with this treatment. Another possibility is that homocysteine is an inflammatory marker, thus associated with low grade inflammation in CHF and that it could be useful to add this marker to follow the progression of CHF.

The results of our study on severely ill CHF patients (NYHA IV) and at high age (mean 85.5 years) were compared to a patient group of (NYHA II) (mean age 76.5 years) and compared to healthy, age matched controls showed, to our surprise, that the vascular reactivity was reduced in CHF but did not correlate with the severity of the condition or age of the patients. An interpretation of this could be that in the early stages of CHF the cascade of neurohormones acting in the circulation, designed to counter-balance with release of different signal molecules, stored in endothelium, nerve fibres, and in the heart and kidney. Perhaps this balancing mechanism takes place early and have already been activated to its limit in the peripheral circulation. Clearly, this “vicious circle” of hormone mechanisms in CHF needs further investigation leading to better understanding and treatment.

As the failing heart syndrome progress, further ways to alleviate the symptoms are required. Hence, the need for new drug therapy is required. BNP *per se* has been used as a last resort treatment in severe CHF. Evidence is not clear to what extent this new therapy could be beneficial for the patients, due to problems with hypotension. To elucidate this issue from our point of view, we designed a study to evaluate the microvascular aspects of BNP in CHF patients. We postulated that the high level of BNP in CHF might lead to a chronic occupation of the local BNP receptors and downregulation of the natriuretic receptor vasodilator response to iontophoretic BNP. This was proven true, thus, the ability of BNP vasodilatory response in CHF patients was reduced to less than half ( $p < 0.01$ ) as compared to the responses in healthy age-matched controls. The relaxant effect of BNP is mediated via the formation of nitric oxide, confirmed by local blockade with L-NAME. The findings are consistent with our hypothesis, that the vascular response to BNP is reduced in CHF patients.

In our future studies we will examine in depth the localization and the mechanisms involved in the natriuretic peptide response in the human microcirculation using myography and molecular biology methods.



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# Paper I



## Original Paper

# High NT-proBNP Is a Strong Predictor of Outcome in Elderly Heart Failure Patients

Sven E. Andersson, MD, PhD;<sup>1</sup> Marie-Louise Edvinsson, BSc;<sup>1</sup> Jonas Björk, PhD;<sup>2</sup> Lars Edvinsson, MD, PhD<sup>1</sup>

From the Department of Emergency Medicine, Institute of Clinical Science,<sup>1</sup> and the Competence Centre for Clinical Research,<sup>2</sup> Lund University Hospital, Lund, Sweden

Address for correspondence: Sven E. Andersson, MD, PhD, Linero Vårdcentral, Vikingavägen 31, S-224 76 Lund, Sweden

E-mail: sven.ea@telia.com

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*All patients older than 65 years (184 men; mean age, 78±0.8 years/181 women; mean age, 82±0.6 years) seeking medical attention at the Lund University Hospital Emergency Clinic during a 2-year period who had an N-terminal prohormone brain natriuretic peptide (NT-proBNP) value >2000 pg/mL were followed up for survival. Mortality in the entire population was 21% after 3 months, 35% after 1 year, and 40% after 2 years. Multivariate analysis indicated that the NT-proBNP level and the New York Heart Association (NYHA) functional class were stronger predictors of mortality than were echocardiographic estimation of left ventricular ejection fraction or chest radiography. Patients who survived the first year were younger, had higher systolic blood pressure, had lower plasma creatinine, had lower inflammatory activity, and were treated with lower doses of furosemide. The results indicate that in this population, NT-proBNP level together with assessment of NYHA class gives the best prognostic information of 1-year mortality. (Am J Geriatr Cardiol. 2008;17:13–20) ©2008 Le Jacq*

It has now been more than 25 years since de Bold and colleagues<sup>1</sup> first injected an atrial extract and observed vasodilator and natriuretic responses, which opened up a new dimension in cardiac physiology. This class of natriuretic peptides has a vital function in cardiovascular homeostasis.<sup>2</sup> In congestive heart failure (CHF), synthesis and release of cardiac natriuretic peptides rise incrementally parallel to reduced cardiac function. The levels of plasma brain natriuretic peptide (BNP) and its amino-terminal residue N-terminal prohormone brain natriuretic peptide (NT-proBNP) correlate positively with cardiac filling volumes, are inversely related to a low left ventricular ejection fraction (LVEF), and are predictors of a CHF diagnosis.<sup>3,4</sup>

CHF is associated with high mortality. Low LVEF, older age, male sex, and presence of diabetes

mellitus are well-recognized prognostic predictors.<sup>5</sup> Epidemiologic studies indicate, however, that in half of the participants, preferentially the elderly and women, CHF is associated with a normal LVEF. Although most studies have found a positive covariation between LVEF and survival, there are reports of similar mortality rates among CHF patients with preserved systolic function as compared with those with reduced cardiac function.<sup>6</sup> Thus, it could be that the prognostic weight of LVEF measurements is reduced in a geriatric population.

Studies in a variety of populations have shown that the plasma level of NT-proBNP predicts mortality, for example, for moderate elevations in a community-based population<sup>7</sup> or in post-myocardial infarction patients.<sup>3</sup> The same association is reported in studies in emergency department patients with dyspnea (the ProBNP Investigation of Dyspnea in the Emergency Department [PRIDE]

study)<sup>8</sup> or in patients with a diagnosis of severe CHF (the Carvedilol Prospective Randomized Cumulative Survival [COPERNICUS] trial).<sup>9</sup> There are, however, few studies performed in geriatric patients, a rapidly growing patient group in the Western part of the world.

The aim of the present study was to examine the long-term prognostic information provided by a single measurement of NT-proBNP in elderly patients admitted with CHF. The prognostic information of NT-proBNP was compared with that of other diagnostic methods like echocardiographic estimates of LVEF, routine radiography, and assessment of New York Heart Association (NYHA) functional class. Furthermore, we established whether factors in the basal characteristics of the patients influenced the prognostic value of NT-proBNP. The results indicated that high NT-proBNP is the strongest indicator of mortality during the first year after measurement.

#### MATERIALS AND METHODS

The study was conducted in patients older than 65 years in whom NT-proBNP was measured and who sought medical attention at the emergency department at Lund University Hospital during 2003 and 2004. The study conforms with the principles outlined in the Declaration of Helsinki and was approved by the Lund University Ethics Committee. The NT-proBNP analyses were performed at the Department of Clinical Chemistry, Lund University Hospital, using the Elecsys system (Roche Diagnostics, Basel, Switzerland). We focused on patients who clearly had CHF. Since there were no data available on the NT-proBNP levels in healthy elderly participants, we performed a prestudy in which we measured the plasma levels of the peptide in 98 healthy persons with a mean age of 79±7 years (range, 65–99 years) who had no clinical signs of CHF. Ninety-five percent of the men in this group had levels <393 pg/mL while the corresponding figure for women was 523 pg/mL. These values could thus be used to rule out CHF. If we instead look for values that could be used for a rule-in decision, Januzzi and associates<sup>8</sup> used 1800 pg/mL as a cutoff for diagnosing CHF in patients older than 75 years and reported that this yielded a high specificity and sensitivity. To further increase specificity, we decided to use a level of 2000 pg/mL as an inclusion criterion. In the present study, 184 men and 181 women met this criterion.

At the time the samples were taken, there was no decision made to compile the data into a study.

All patients were thus treated according to clinical routine with normal diagnostic procedures and clinical chemistry assessments. The patients were followed up in the general population register for survival during the 2 years after the inclusion.

Januzzi and colleagues<sup>8</sup> reported a distinct difference in survival rates between patients with plasma NT-proBNP concentrations >5000 pg/mL and those with concentrations <5000 pg/mL during the first 3 months after measurement. In our material, this dichotomy was less evident and the patients were instead allocated to 4 groups according to NT-proBNP level: 2000 to 3000 pg/mL (n=30); 3000 to 5000 pg/mL (n=101); 5000 to 10,000 pg/mL (n=99); and >10,000 pg/mL (n=135).

The routine at our clinic is that all patients with suspected CHF should undergo echocardiographic examination. They are, however, not regularly followed up with repeated examinations. In the present study, 82 female (45%) and 116 male (63%) patients were investigated within 2 weeks of the NT-proBNP measurements. The investigations were performed at the Department of Cardiology, Lund University Hospital, by a specialist in echocardiography. The visual estimates of the LVEF were included. These data were often given semiquantitatively; so the results were translated as follows: normal, LVEF >50%; mildly reduced, LVEF 50% to 40%; moderately reduced, LVEF 40% to 30%; and severely reduced, LVEF <30%.

Chest radiography was performed the same day or within a few days before or after the NT-proBNP sampling in 324 (89%) of the patients. For the analysis in this study, we divided the participants into 2 groups: those with any sign of decompensated CHF as judged by the radiologist (eg, distended vessels, interstitial edema, pleural effusion, and pulmonary edema) and those with no sign thereof.

NYHA functional class was determined by the treating physician and documented in a few patients (<5%) in the patient records. For the rest of the patients, however, this was not the case, and for all of these an estimate was made based on the information on physical ability registered in the physician's or nurse's medical records from the day of admission. The criteria for NYHA class IV were dyspnea at rest or when talking, need for oxygen, presence of rales, and anxiety. These patients typically arrived at the hospital by ambulance. For NYHA class III, the criteria were inability to walk distances more than a few hundred meters, declive edemas, and complaints of reduced physical capacity. Patients judged to be in NYHA class II typically entered the emergency

department with a written referral from a primary care physician who had noticed a moderate reduction of physical capacity.

Electrocardiography was routinely performed in conjunction with admission and results were stored in a central database. In the evaluation, these could be found for all but 16 patients. The electrocardiograms were evaluated separately for rhythm (sinus, atrial fibrillation, or pacemaker), bundle branch block (left or right), and signs of left ventricular hypertrophy by a computer program and a specialist in internal medicine.

The actual medication taken on the day of the NT-proBNP sampling was recorded. The administered doses of furosemide, spironolactone,  $\beta$ -adrenergic receptor antagonists, and angiotensin-converting enzyme (ACE) inhibitors/angiotensin II receptor blockers (ARBs) are presented. For the 2 former, the doses in milligrams were used in the analyses; for the 2 latter, the daily dose recommended for the treatment of CHF for the individual substance was used. For  $\beta$ -adrenergic receptor blockers, the values were as follows: 200 mg metoprolol, 100 mg atenolol, 10 mg bisoprolol, 50 mg carvedilol (<85 kg), and 100 mg carvedilol (>85 kg). For ACE inhibitors/ARBs, the doses were 10 mg ramipril, 100 mg captopril, 32 mg candesartan, 20 mg enalapril, 35 mg lisinopril, 50 mg losartan, and 300 mg irbesartan.

**Statistical Analysis.** All statistical analyses were conducted using SPSS 12.0.1 for Windows (SPSS Inc, Chicago, IL) with  $P < .05$  as a significance level. Since the majority of deaths occurred within the first year after measurement of NT-proBNP, the statistical difference between 1-year survivors and nonsurvivors was calculated for most measured parameters. The effects of NT-proBNP together with sex, age, radiographic diagnosis, LVEF, NYHA functional class, pharmacologic treatment, and echocardiographic pattern (rhythm, bundle branch block, and left ventricle hypertrophy) on mortality within 12 months were evaluated in univariate and multivariate analyses using Cox regression. In the analyses, we categorized LVEF as mentioned above, age (younger than 70 years, 71–80 years, 81–85 years, or older than 85 years), and radiographic diagnostics (CHF vs no CHF). The effect of NT-proBNP was evaluated as a continuous variable, transformed using  $\log_2$ , and categorized as above. The medications were furosemide (dosage categorized as 0, 1–50, 51–100, or >100 mg/d), spironolactone (dosage categorized as 0, 1–25, or >25 mg/d), and  $\beta$ -adrenergic

receptor blockers (dosage categorized as 0, 1–25, 26–50, or >50% of recommended dose), and ACE inhibitors/ARBs (dosage categorized as 0, 1–50, or >50% of recommended dose). All other measures were entered as continuous variables. NYHA class I was small ( $n=2$ ) and was therefore collapsed with NYHA class II. A low NYHA class (I or II) was strongly related to survival, with only 2 deaths within 12 months among 47 participants. All analyses were therefore both conducted for all patients and restricted to NYHA class III and IV ( $n=318$ ). In the analyses, we first evaluated the effect of each variable separately on mortality. We then established 2 different multivariate Cox regression models. In the first model, NT-proBNP (continuous) together with all variables with  $P < .30$  in the univariate analyses except NYHA class, LVEF, and radiographic findings were entered in a multiple Cox regression model. From this, we excluded one insignificant independent variable at a time, starting with the variable with highest  $P$  value, until only statistically significant predictors of mortality remained. For the categorical variables, the  $P$  value for the overall Wald statistic was used. For patients who did not undergo echocardiography and radiography, we added separate unknown categories for these 2 variables in the multivariate analyses. In the second model, we included the diagnostic methods NT-proBNP (categorized), NYHA class, LVEF, and radiographic findings as independent variables.

## RESULTS

### NT-proBNP Values in Healthy Elderly Persons.

The mean plasma NT-proBNP level in this group was  $241 \pm 22$  pg/mL in women and  $171 \pm 20$  in men ( $P < .05$ ). There was a low but significant positive correlation between age and peptide level ( $r=0.22$ ).

### Mortality and the Predictive Value of the Different Diagnostic Methods.

Mortality in the entire CHF group was high. At 3 months, 76 patients (21%) had died. Corresponding values for 1 and 2 years were 127 (35%) and 146 (40%), respectively. The survival time was negatively associated with NT-proBNP value. For patients in the group with the highest NT-proBNP levels (>10,000 pg/mL), there was a particularly high mortality rate within the first 100 days. After this the mortality rate was slowly declining without any abrupt jumps (Figure 1).

The assessed NYHA class increased in tandem with the NT-proBNP value, and there was a significant positive correlation between NYHA class and mortality (Figure 2, Table I).

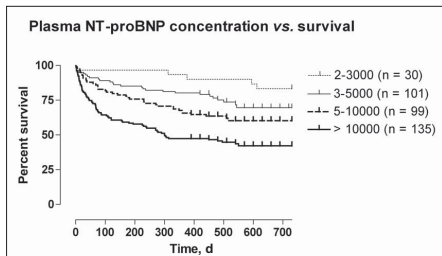


Figure 1. Kaplan-Meier curve for survival after measurement of N-terminal pro-hormone brain natriuretic peptide (NT-proBNP). The population is divided into 4 groups according to NT-proBNP value: 2–3000 pg/mL, 3–5000 pg/mL, 5–10,000 pg/mL, and >10,000 pg/mL. NT-proBNP value was negatively associated with survival ( $P < .001$ )

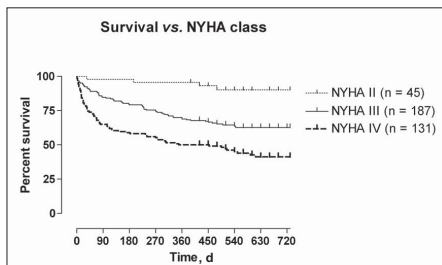


Figure 2. Kaplan-Meier curve for survival after measurement of N-terminal pro-hormone brain natriuretic peptide in patients with different New York Heart Association (NYHA) functional class. NYHA class was negatively associated with survival ( $P < .001$ ).

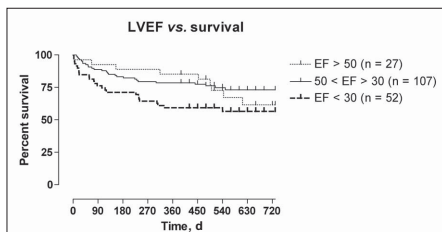


Figure 3. Kaplan-Meier curve for survival after measurement of N-terminal pro-hormone brain natriuretic peptide in patients with different levels of left ventricular ejection fraction (LVEF). In the entire material, LVEF was inversely associated with mortality ( $P = .03$ ). EF indicates ejection fraction.

In all of the material, LVEF was inversely associated with mortality ( $P = .03$ ; Figure 3). A similar tendency ( $P = .07$ ) was seen when restricting the analysis to patients in NYHA classes III and IV.

LVEF did not correlate significantly with the NT-proBNP value, however.

In the 1-year survival group, 51% of the radiographs obtained showed findings positive for CHF, while the corresponding figure for nonsurvivors was 71%. In the univariate analysis, this difference is significant for the entire population ( $P = .003$ ) and also when restricted to NYHA classes III and IV ( $P = .03$ ). In patients with positive radiographic findings, the NT-proBNP values were significantly higher (median, 8956; first-third quartile 4679–19,357) than in those with no sign of incompensation (median, 5723; first-third quartile, 3722–12,443;  $P = .001$ ; Mann-Whitney U test).

**Patient Characteristics, Pharmacologic Treatment, and Association With Mortality.** Baseline characteristics are given in Table I. In the univariate analyses among all participants, significant associations with mortality within 12 months were observed for age, plasma creatinine level, systolic blood pressure (BP), and C-reactive protein (CRP) level.

There was no significant sex difference in median NT-proBNP values ( $P = .20$ ; Mann-Whitney U test), but there was a small albeit statistically significant difference in age (median in men, 83 years vs 80 years in women;  $P < .001$ ; Mann-Whitney U test). The prognosis was similar in men and women. Thus, at 3 months, 20% of the men and 22% of the women had died. The corresponding figures for 1 year were 35% and 34%, respectively. These figures should be compared, however, with the expected yearly mortality for these age groups in our region, which was 9% in men and 3% in women for the actual years.<sup>10</sup> At the 2-year follow-up, the mortality rate was 40% in both sexes.

In only 7 patients (2%), the electrocardiographic recordings were considered normal. Of those who survived the first year, 38% had atrial fibrillation; 12%, pacemaker rhythm; and the remaining, sinus rhythm. The frequencies were similar in the nonsurvival group (34% atrial fibrillation and 13% pacemaker). Bundle branch block was found in 23% of the survivors vs 33% of the nonsurvivors. Twenty-seven percent of the survivors and 23% of the nonsurvivors had signs of left ventricular hypertrophy. None of these differences were significant in the univariate analysis.

The pharmacologic treatment on the day of NT-proBNP measurement differed somewhat between survivors and nonsurvivors. The median dose of furosemide was 80 mg in both groups, with the 25th to 75th percentiles being 40 to 90 mg in the former and 40 to 120 mg in the latter.

|                                 | SURVIVORS (N=238)  | NONSURVIVORS (N=127) | P VALUE | P VALUE RESTRICTED TO NYHA CLASS III OR IV |
|---------------------------------|--------------------|----------------------|---------|--|
| Male/female, No.                | 119/119            | 65/62                | >.30    | >.30                                       |
| Age, y                          | 80 (73–85)         | 83 (78–88)           | .01     | .04  |
| NT-proBNP, pg/mL                | 5734 (3696–10,966) | 11,668 (6337–28,605) | <.001   | <.001                                      |
| NYHA functional class           | III (III–IV)       | IV (III–IV)          | <.001   | <.001                                      |
| Body weight, kg                 | 74 (60–85)         | 68 (55–79)           | .26     | .29  |
| Systolic blood pressure, mm Hg  | 140 (120–151)      | 120 (108–140)        | <.001   | <.001                                      |
| Diastolic blood pressure, mm Hg | 75 (60–85)         | 70 (60–80)           | .08     | .16  |
| Plasma creatinine, $\mu$ M      | 91 (72–125)        | 119 (84–173)         | <.001   | <.001                                      |
| Hemoglobin, g/L                 | 124 (109–137)      | 120 (107–130)        | .11     | .39  |
| CRP, mg/L                       | 17 (8–46)          | 33 (11–72)           | .009    | .02  |

<sup>a</sup>Values are given as median (25th and 75th percentiles) or as frequency. <sup>b</sup>Values for body weight could not be found for 10% of the population. Abbreviations: CRP, C-reactive protein; NT-proBNP, N-terminal prohormone brain natriuretic peptide; NYHA, New York Heart Association.

|                                 | HAZARD RATIO | 95% CONFIDENCE INTERVAL | P VALUE |
|---------------------------------|--------------|-------------------------|---------|
| Log <sub>2</sub> (NT-proBNP)    | 1.6          | 1.4–1.9                 | <.001   |
| Age, y                          |              |                         | .03     |
| 70 or younger                   | 1.0          | –                       | –       |
| 71–80                           | 1.9          | 0.90–4.0                | .09     |
| 81–85                           | 2.6          | 1.3–5.5                 | .01     |
| 86 or older                     | 2.7          | 1.3–5.6                 | .009    |
| Systolic blood pressure         | 0.98         | 0.97–0.99               | <.001   |
| Furosemide, mg/d                |              |                         | .02     |
| 0                               | 1.0          | –                       | –       |
| 1–50                            | 0.91         | 0.48–1.7                | >.30    |
| 51–100                          | 0.74         | 0.40–1.4                | >.30    |
| >100                            | 1.4          | 0.80–2.6                | .22     |
| ACE inhibitors/ARBs, total mg/d |              |                         | .04     |
| 0                               | 1.0          | –                       | –       |
| 1–50                            | 0.56         | 0.36–0.87               | .01     |
| >50                             | 0.90         | 0.53–1.5                | >.30    |

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; NT-proBNP, N-terminal prohormone brain natriuretic peptide

One-year survival was significantly associated with administration of lower amounts of furosemide in the univariate analyses ( $P=.001$ ). Restricting the analysis to NYHA classes III and IV did not alter this significance ( $P=.005$ ).

About 40% of the patients received  $\beta$ -adrenergic receptor blockers in a median dose of 25% of the recommended dose (25–50 mg). Forty-five percent of the survivors and 33% of the nonsurvivors were treated with ACE inhibitors/ARBs. The median dose was 50% of the recommended dose for both groups. Spironolactone was administered to 67% of

the survivors and 59% of the nonsurvivors. Some associations with survival were also indicated for these other medications, but none of these reached significance in the univariate analyses ( $P\geq.06$ ).

**Multivariate Analysis.** In the multivariate model where risk factors but no diagnostic methods except NT-proBNP were included, older age and low systolic BP were associated with increased mortality (Table II). The associations between the medications and mortality were generally weak and exhibited no consistent dose-response relationship. According to

**Table III. Multivariate Associations Between Diagnostic Results and Survival**

|                  | ALL PATIENTS |          |         | NYHA CLASS III, IV |          |         |
|------------------|--------------|----------|---------|--------------------|----------|---------|
|                  | HR           | 95% CI   | P VALUE | HR                 | 95% CI   | P VALUE |
| NT-proBNP, pg/mL |              |          | .001    |                    |          | .002    |
| <3000            | 1.0          | –        | –       | 1.0                | –        | –       |
| 3001–5000        | 3.4          | 0.79–15  | .10     | 3.4                | 0.78–15  | .10     |
| 5001–10,000      | 4.5          | 1.1–19   | .04     | 4.5                | 1.1–19   | .04     |
| >10,000          | 7.4          | 1.8–30   | .006    | 7.0                | 1.7–29   | .007    |
| NYHA class       |              |          | <.001   |                    |          | .002    |
| I, II            | 1.0          | –        | –       | –                  | –        | –       |
| III              | 4.8          | 1.5–15   | .01     | 1.0                | –        | –       |
| IV               | 8.4          | 2.6–28   | <.001   | 1.8                | 1.2–2.6  | .002    |
| LVEF             |              |          | <.001   |                    |          | <.001   |
| >50%             | 1.0          | –        | –       | 1.0                | –        | –       |
| 41%–50%          | 1.5          | 0.47–4.5 | >.30    | 1.3                | 0.43–4.3 | >.30    |
| 31%–40%          | 1.1          | 0.34–3.5 | >.30    | 1.1                | 0.34–3.4 | >.30    |
| <30%             | 2.5          | 0.85–7.2 | .10     | 2.4                | 0.84–7.1 | .10     |
| Unknown          | 4.0          | 1.5–11   | .007    | 4.0                | 1.4–11   | .008    |
| Chest radiology  |              |          | .11     |                    |          | .11     |
| Negative         | 1.0          | –        | –       | 1.0                | –        | –       |
| Positive         | 1.4          | 0.96–2.2 | .08     | 1.4                | 0.96–2.2 | .08     |
| Unknown          | 0.88         | 0.43–1.8 | >.30    | 0.85               | 0.41–1.8 | >.30    |

Cox regression model for the effect of N-terminal prohormone brain natriuretic peptide (NT-proBNP), New York Heart Association (NYHA) class, left ventricular ejection fraction, and chest radiography on 1-year mortality among patients with congestive heart failure. Models were established for all patients (N=365) and restricted to NYHA classes III and IV (n=318). Abbreviations: CI, confidence interval; HR, hazard ratio; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal prohormone brain natriuretic peptide; NYHA, New York Heart Association.

the model, doubling the NT-proBNP level increased the mortality risk by 60% (hazard ratio [HR], 1.6; 95% confidence interval [CI], 1.4–1.9). A similar model was obtained when the analysis was restricted to NYHA classes III and IV (data not shown). In the 4-variable multivariate model for the diagnostic methods, NT-proBNP and NYHA class were both strongly related to survival (Table III). The effect of NT-proBNP was of similar magnitude both among all patients and when restricting the analysis to those with NYHA classes III and IV. The 1-year risk was approximately 7 times higher among participants with NT-proBNP level >10,000 pg/mL as compared with participants with NT-proBNP level ≤3000 pg/mL. Survival in NYHA classes III and IV also differed significantly (HR, 1.8; 95% CI, 1.2–2.6). The association between LVEF and survival was less strong and only significant for the unknown category. Chest radiography results were not significantly related to survival.

## DISCUSSION

This study shows that the level of NT-proBNP increases slightly in elderly persons and that marked elevations

of the levels are strong predictors of mortality in a geriatric population. In the healthy group, women had significantly higher values than men; this sex difference was lost in the CHF population, however.

In patients with CHF, the mortality rate was even higher than that found in the classic Framingham study, in which 37% of the men and 34% of the women died within 2 years of a positive CHF diagnosis.<sup>11</sup> It could be that our population was older and affected with more advanced forms of disease, but still the results indicate that the high mortality in clinically manifested CHF has not improved during the last 2 decades despite improvement in diagnostic methods and pharmacotherapy.

The survival curves show an interesting time pattern in which mortality, particularly for the group with the highest NT-proBNP values, was higher within the first months after the measurement than it was later in the course. After 1 year, the mortality approached background values, which suggests that the prognostic information of a single measurement of NT-proBNP did not extend beyond the 1-year time point. The leveling off by the curves could have several explanations.

One is that the NT-proBNP value can vary considerably over time because of success in treatment. It could be that among the patients who survived, the condition of a large portion had improved and thus their values were reduced over time.<sup>12</sup>

A low CRP level was univariately associated with good prognosis; CHF is a well-known inflammatory condition, and elevated CRP is associated with high NT-proBNP level.<sup>13</sup> Nonsurvivors also had higher plasma creatinine values, which suggests more reduced renal function. Again, this could be a part of CHF, since impaired renal function is associated with CHF.<sup>14</sup>

The main difference between the 1-year survivors and nonsurvivors in the univariate analyses of pharmacologic treatments was that the survivors were given lower amounts of furosemide. This is in agreement with other reports<sup>15</sup> but is likely to be another indication of the severity of the condition, which is likely to correlate with more intense treatment. Fewer than half of the patients were treated with ACE inhibitors/ARBs and  $\beta$ -adrenergic blockade. In those treated, the doses used were far from those recommended. This did not differ significantly between survivors and nonsurvivors. The medication was recorded on the day the NT-proBNP sample was taken, which usually was at the beginning of the hospital stay. For many patients, the medication was increased in the period after inclusion. But as can be seen from the BP and creatinine values, particularly in the nonsurvivor group, there was limited possibility to increase the medical treatment much further.

In the multivariate analysis of risk factors together with NT-proBNP level, the latter was the most powerful predictor of 1-year mortality, followed by age and low systolic BP. This supports the conclusion that several of the other parameters found significant in the univariate analysis reflect the severity of CHF and thus were no longer significant when NT-proBNP level was included in the analysis. The correlation between age and mortality is natural. The association between a higher systolic BP and survival is likely to be another reflection of the severity of the condition since a low cardiac output and/or the requirement of intense medical treatment would reduce BP. Interestingly, sex did not influence the prognosis in this population. When we conducted a multivariate adjustment for the other prognostic factors of importance, including age and NT-proBNP level, sex remained insignificant ( $P > .30$ ). This is in contrast with another report,<sup>5</sup> in which there were better outcomes in women. A possible interpretation is that the strong

prognostic impact of NT-proBNP in our population overruns that of sex.

We then compared the prognostic information provided by NT-proBNP level to that provided by other diagnostic methods and observed that both NYHA class and the presence of positive radiologic findings were associated with higher NT-proBNP values. Estimated LVEF, however, was not associated. A likely explanation for this is that a large portion of our patients had CHF with preserved systolic function, which is reported to be more common in the elderly.<sup>6</sup> In the univariate analysis, the results of all these diagnostic methods were associated with mortality. The association was strongest with NYHA classification and remained significant in the multivariate analysis. It is of clinical interest to note that this simple classification could bear such an amount of information. These results are in agreement with those of others since the combination of NYHA classification and measurement of BNP gives a good estimate of CHF severity and aerobic exercise capacity.<sup>16</sup>

The associations between mortality and LVEF and radiography were weaker and not significant in the multivariate analysis of the diagnostic methods. For LVEF, mortality was only significantly elevated among participants in whom no measurement was performed, which might suggest a bias. It seems likely that this investigation was omitted in patients with a very short remaining life expectancy. Several patients did not have a recent echocardiographic examination. Therefore, firm conclusions are difficult to draw, but it seems important to focus more on the presence of diastolic dysfunction when the result of echocardiography is interpreted in elderly patients. From the radiographic analysis, it can be concluded that since half of the 1-year survivors had positive findings, this investigation provides little prognostic information of practical value.

The absolute majority of our patients had pathologic electrocardiographic results. The abnormalities were not, however, associated with the prognosis. It is natural that such a widespread parameter cannot provide this type of information. Our results are also in agreement with the reported finding that left bundle branch block per se does not appear to be an important independent predictor of mortality in myocardial infarction patients.<sup>17</sup>

## CONCLUSIONS

Our results suggest that mortality in elderly CHF patients is determined by the severity of cardiac condition and age. NT-proBNP level and NYHA classification bear the best prognostic information, but this is limited to the first year after measurement.

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# Paper II



# Cutaneous vascular reactivity is reduced in aging and in heart failure: association with inflammation

S. E. ANDERSSON, M.-L. EDVINSSON and L. EDVINSSON

Department of Internal Medicine, Lund University Hospital, S-226 85 Lund, Sweden

## A B S T R A C T

In the present study, we have investigated whether changes in vascular reactivity in congestive heart failure (CHF) patients can be detected in the cutaneous microvessels and whether these changes are due to endothelial dysfunction, are affected by increasing age and related to an ongoing inflammation. The responses to local warming and iontophoretically administered endothelium-dependent and -independent vasodilators were investigated in healthy young adults, healthy elderly adults and elderly adults with CHF. The results were correlated with plasma concentrations of vascular risk factors and markers for endothelial dysfunction and inflammation. The vasorelaxant responses were reduced in the elderly groups and were attenuated further in the CHF group. This group also had increases in levels of several markers associated with inflammation, higher blood glucose and homocysteine levels, a lower low-density lipoprotein–cholesterol and a rise in the concentration of von Willebrand factor, indicating a prothrombotic endothelial function. The severity of the heart failure, measured as the plasma level of brain natriuretic peptide, correlated with the intensity of inflammation and to the changes in vascular risk factors and endothelial function. It is concluded that the reactivity of the cutaneous microvessels is reduced with age, and the presence of CHF causes a further impairment. There is endothelial dysfunction in CHF, but it is uncertain to what extent this contributes to the reduced vasodilatory capacity. The inflammatory response appears central for many of the manifestations of the CHF syndrome.

## INTRODUCTION

Congestive heart failure (CHF) is a clinical syndrome characterized by a recognizable pattern of haemodynamic, renal, hormonal and neural responses [1]. Several of the compensatory mechanisms affect the peripheral circulation. A common finding is that the vasodilatory response to increased metabolic demands and vasorelaxing autacoids is reduced. Since the vasorelaxant capacity is important for the control of tissue perfusion,

an impairment could contribute to several of the patient's symptoms, such as exaggerated muscle hypoxia during exercise. When these abnormalities affect the coronary circulation, they can further augment the myocardial damage. Furthermore, the total peripheral vascular resistance will tend to rise, thus increasing the workload of the heart [2]. It has been proposed that the impaired vasodilation is mainly due to a change in the functional mode of the endothelium, with a reduction in local concentrations of endothelium-derived

**Key words:** aging, circulatory physiology, endothelial function, heart failure, inflammation, thrombosis, vasculature.

**Abbreviations:** ACh, acetylcholine; AUC, area under the curve; BMI, body mass index; BNP, brain natriuretic peptide; CHF, congestive heart failure; CRP, C-reactive protein; EDF endothelial dysfunction; Hcy, homocysteine; IGT, impaired glucose tolerance; IL, interleukin; LDL, low-density lipoprotein; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species; sIL-2r, soluble IL-2 receptor; SNP, sodium nitroprusside; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; vWf, von Willebrand factor; XO, xanthine oxidase.

**Correspondence:** Dr Sven E. Andersson (e-mail [Sven.Andersson@skane.se](mailto:Sven.Andersson@skane.se)).

vasodilators such as nitric oxide (NO), adenosine or endothelium-derived hyperpolarizing factor (EDHF) [3–5]. This has been denoted as endothelial dysfunction (EDF). Inflammation can induce EDF [6], possibly by enhancing the production of reactive oxygen species (ROS), which can scavenge NO [7]. In CHF, the plasma levels of some pro-inflammatory mediators are elevated, indicating a low-grade inflammatory reaction [4]. It has thus been hypothesized that EDF is a part of the CHF syndrome and secondary to inflammation [3,4]. There are, however, contradictory data, which suggest that the reduced vasodilatory capacity is attributed to non-endothelial factors such as an increased vasoconstrictive tone at rest or to changes in vascular smooth muscle function ‘vascular stiffness’ [8].

It has been postulated that there are two principal modes of endothelial behaviour [9]. First, in its normal ‘antithrombotic’ state, the endothelium mediates vasodilation, prevents platelet adhesion and activation, and blocks thrombin formation. Adhesion and transmigration of leucocytes are attenuated and ROS are effectively scavenged. Secondly, when the endothelium is disturbed, for example by inflammation, completely opposite actions occur. In this dysfunctional state, there is vasoconstriction, increased endothelial production of von Willebrand factor (vWf), plasminogen activator inhibitor-1 (PAI-1), ROS and various adhesion molecules. This is thus a wider meaning of the term EDF. Reports of an elevated risk for thromboembolic events suggest that the endothelium could be in this ‘prothrombotic state’ in CHF [10].

There are several methods to quantify endothelium-dependent vasodilation [11]. The most common is the invasive forearm technique where blood flow changes are measured by venous occlusion plethysmography after challenge with endothelium-dependent and -independent dilators. In the present study, we investigated if a non-invasive technique could be suitable for studies in this area instead: blood flow changes were induced by local iontophoretic administration of vasodilator drugs to the skin. We measured if the vasodilatory responses in this vascular bed were reduced in CHF and if this was due to EDF. Furthermore, we wanted to examine if there is a correlation between changes in vascular function, inflammatory activity, severity of CHF and prothrombotic activity. Since the vasodilatory capacity is reported to decline with increasing age [12], we also wanted to study the influence of this parameter.

## MATERIALS AND METHODS

### Ethics

The investigation conforms with the principles outlined in the Declaration of Helsinki. The Ethics Committee

**Table 1** Underlying disease judged as the cause for CHF

Two of the patients with ischaemic disease also had additional complications (hypertension and dilated cardiomyopathy respectively). A patient with valvular disease also had toxic cardiomyopathy.

| Underlying disease                      | <i>n</i> |
|---|----------|
| Ischemic heart disease                  | 8        |
| Dilated cardiomyopathy                  | 2        |
| Toxic cardiomyopathy                    | 2        |
| Hypertrophic obstructive cardiomyopathy | 1        |
| Hypertension                            | 2        |
| Valvular disease                        | 2        |
| Unknown                                 | 1        |

of Lund University approved the protocol (LU 395-00). Informed consent was obtained from all subjects.

### Subjects

Studies were performed on three groups. Group 1: 15 patients (ten men and five women) treated at Lund University Hospital for CHF. They had a reduced left ventricular function upon echocardiography and elevated plasma levels of brain natriuretic peptide (BNP). Patients with diabetes mellitus, uraemia, medication with long-acting nitrates, oxygen treatment, ongoing infection, dementia, active smoking habit or the presence of tremor were excluded. Two patients were judged to be in New York Heart Association functional class II, twelve in class III and one in class IV. Five patients had atrial fibrillation, one had a pacemaker and the remainder had sinus rhythm. The assessed causes of CHF in these patients are given in Table 1. Group 2: healthy age- and gender-matched controls to group 1. These subjects were not allowed to have clinical signs of CHF or plasma levels of BNP exceeding 25 pmol/l. In this group, one male subject was excluded because of elevated BNP. Group 3: in order to study the influence of age on vascular reactivity, six healthy young adults (three women and three men) were also studied. No blood samples were taken from this group of subjects. Basal characteristics for the subjects are given in Table 2.

### Pharmacological treatment

At the time of the study, all of the patients were treated with diuretics. In addition, seven patients were on medication with digitalis, two were taking calcium channel blockers, eight were taking angiotensin-converting-enzyme (ACE)-inhibitors, eight were taking  $\beta$ -adrenergic antagonists, seven were taking warfarin, three were taking allopurinol, two were taking statins, eight were taking salicylic acid, three received vitamin B<sub>12</sub> and two received folic acid. In the age-matched control group, two subjects were taking diuretics, three were taking  $\beta$ -adrenergic

**Table 2** Clinical characteristics of the elderly CHF patients and young and elderly controls

MAP, mean arterial pressure. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with elderly controls, as determined by the Kruskal–Wallis/Mann–Whitney  $U$  test. Q, regional blood flow.

| Parameters                             | Young controls | Elderly controls | Elderly CHF  |
|--|----------------|------------------|--------------|
| Age (years)                            | 23 ± 2         | 74 ± 2           | 74 ± 2       |
| Heart frequency (bpm)                  | 64 ± 1         | 64 ± 2           | 81 ± 3***    |
| MAP (mmHg)                             | 72 ± 4**       | 96 ± 3           | 94 ± 4       |
| Pulse pressure (mmHg)                  | 48 ± 2         | 55 ± 5           | 47 ± 3       |
| Body temperature (°C)                  | 37.0 ± 0.1     | 37.0 ± 0.1       | 37.1 ± 0.2   |
| Skin temperature (°C)                  | 30.1 ± 0.3*    | 28.6 ± 0.2       | 30.2 ± 0.5** |
| BMI (kg/m <sup>2</sup> )               | 22 ± 1*        | 26 ± 1           | 24 ± 1       |
| Serum creatinine (mg/l)                | Not measured   | 75 ± 2           | 90 ± 7       |
| Serum BNP (pmol/l)                     | Not measured   | 8.0 ± 1.8        | 198 ± 43***  |
| Cutaneous vascular resistance (mmHg/Q) | 12.3 ± 2.1     | 12.7 ± 1.0       | 10.7 ± 1.3   |

blockers and three were taking salicylic acid. None of the young adults was on any medication.

### Quantification of vascular risk factors

The vascular risk factors homocysteine (Hcy), glucose, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein-cholesterol were deemed likely to influence the vascular reactivity. The plasma levels were thus measured in the CHF patients and their controls.

### Estimation of inflammatory and prothrombotic activity

As markers for inflammatory activity, we chose to quantify the plasma levels of C-reactive protein (CRP; high-sensitive method), interleukin (IL)-6, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), soluble IL-2 receptor (sIL-2r), IL-1 $\beta$  and IL-8.

CHF has been described as a hyperuricaemic condition [13], and the serum uric acid levels have been found to strongly correlate with inflammatory activity [14]. Uric acid is thus regarded as a marker of chronic inflammation. vWf and PAI-1 were measured as markers for a prothrombotic state of the endothelium. vWf and PAI-1 concentrations were analysed at the Department of Clinical Chemistry, Malmö University Hospital, Malmö. All other blood samples were analysed at the Department of Clinical Chemistry, Lund University Hospital.

### Blood flow measurements

Cutaneous blood flow was measured using the PeriFlux System 5000 (Perimed, Järfälla, Sweden). This method is non-invasive and gives minimal discomfort to the studied subject. Laser-generated light at a wavelength of 780 nm is directed to the skin using a fibre optic probe. The light reflected from moving blood cells in the superficial skin

microvessels undergoes a shift in frequency (Doppler effect) that is proportional to the number and velocity of the moving blood cells. The laser Doppler output is semi-quantitative and we have presented all data as the percentage change compared with the baseline perfusion value. Temperature of the skin was recorded continuously. The ratio between blood pressure (mmHg) and the value for the local blood flow given by the Doppler output was calculated as an estimate of the basal cutaneous vascular resistance.

### Iontophoresis

Constant current iontophoresis was used to enhance the perfusion of charged molecules into the skin of the dorsal side of the lower arm. Endothelium-dependent vasodilation was provoked by iontophoresis of the cation acetylcholine (ACh; 2% dissolved in MilliQ water; Sigma, St. Louis, MO, U.S.A.) using anodal current. Endothelium-independent vasodilation was provoked by iontophoresis of the negatively charged NO donor sodium nitroprusside (SNP; 1% in MilliQ water; Sigma) using cathodal current.

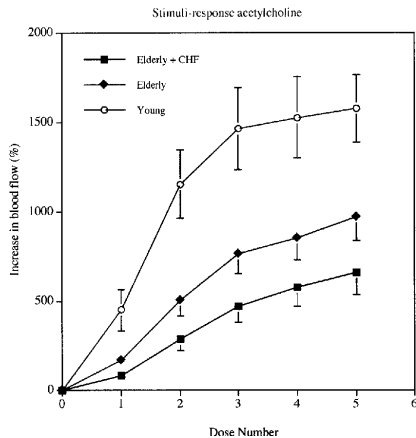
The PeriFlux System (Perimed) used in this study contains an applicator with a small recess in the centre and circular temperature probe surrounding the application site. The recess in the centre allows the insertion of a fibre optic probe to measure the blood flow in the stimulated area. An additional temperature probe containing a fibre optic probe was placed at a distance suitable to avoid large veins. This was used as a reference during the iontophoresis and was subsequently used to determine the response to local warming.

### Protocol

All studies were performed in a temperature-controlled room at 22–24°C. All subjects were resting in the supine position. Blood pressure and heart rate were measured before and after stimulation, and the lowest value is given. The skin of the lower arm was gently cleaned with an alcohol swab and the iontophoretic applicators/fibre optic probes were applied to the forearm. The basal blood flow was studied for 2 min after which ACh was transferred by iontophoresis (anodal current, 0.2 mA for 20 s). The current alone did not affect the in blood flow (results not shown). Repeating the iontophoretic stimulation five times at 60 s intervals produced a stimulus-response curve. Endothelium-independent vasodilation was studied by iontophoresis of SNP as above (cathodal current, 0.1 mA for 60 s). The stimulation was repeated four times at 60 s intervals. Finally, the response to heat was measured following local warming to +44°C for 10 min.

### Statistical analysis

Statistical analysis was performed by Mann–Whitney  $U$  test when two groups were compared, or by



**Figure 1** Stimuli-response curves for changes in cutaneous blood flow following iontophoretic application of ACh to CHF patients, age-matched controls and young controls

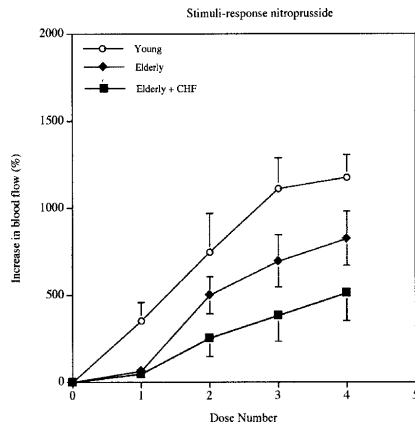
Elderly CHF patients ( $n = 15$ ) and age-matched controls ( $n = 14$ ) had a mean age of 74 years, whereas young controls ( $n = 6$ ) had a mean age of 23 years. Values for the changes in cutaneous blood flow are expressed as a percentage of the baseline and are means  $\pm$  S.E.M. AUC values for elderly controls was statistically different from both young controls ( $P = 0.011$ ) and CHF patients ( $P = 0.026$ ) as determined by Kruskal–Wallis/Mann–Whitney tests.

Kruskal–Wallis test, followed by Mann–Whitney when there were more than two groups. For analysis of the stimuli-response curves an estimate for the area under the curve (AUC) was performed for each patient by adding the values for each stimulation. The resulting numbers were then used for the determination of statistical significance. This also reduced the random variability between different measurements [15]. Calculations were performed using StatView 5.0 (StatView, Berkeley, CA, U.S.A.).

## RESULTS

### Vascular reactivity

The estimated cutaneous vascular resistance did not differ significantly between the groups before stimulation (Table 2). The vasodilatory response to ACh, measured as the AUC, was significantly higher in the young adults than in the healthy elderly controls (Figure 1). The AUC values (mean  $\pm$  S.E.M.) were  $3251 \pm 456$  for the elderly controls,  $2002 \pm 378$  for the CHF patients and  $6195 \pm 887$  for the young controls ( $P = 0.026$  and  $P = 0.011$  respectively, compared with elderly controls). Also, the response to SNP was higher in young compared with elderly controls, but the difference did not reach



**Figure 2** Stimuli-response curves for changes in cutaneous blood flow following iontophoretic application of SNP to CHF patients, age-matched controls and young controls

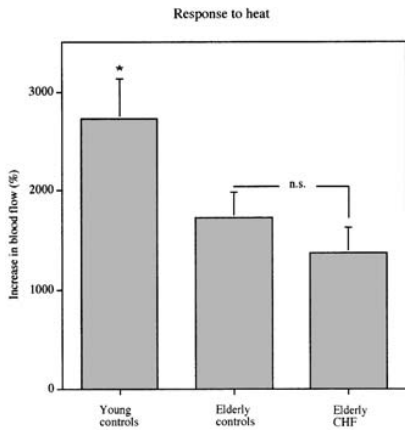
CHF patients ( $n = 15$ ), age-matched controls ( $n = 14$ ) and young controls ( $n = 6$ ). Values for the changes in cutaneous blood flow are expressed as a percentage of the baseline and are means  $\pm$  S.E.M. AUC values for elderly controls statistically different from CHF patients ( $P = 0.02$ ; Kruskal–Wallis/Mann–Whitney tests). Difference from young controls did not reach statistical significance.

statistical significance. The effect of SNP was significantly attenuated in CHF patients compared with the age-matched controls (Figure 2). Values were  $2230 \pm 397$  for the elderly controls,  $1187 \pm 198$  for the CHF patients ( $P = 0.02$  compared with age-matched controls) and  $3014 \pm 384$  for the young controls (not significant compared with elderly controls).

The response to heat was higher in the young adults compared with the elderly controls and CHF patients. There was no difference between the two elderly groups in this respect. The response to heat was greater in magnitude than the highest response following pharmacological stimulation (Figure 3). The increase in blood flow following ACh, expressed as increase in the percentage of the response to heat, was  $58.1 \pm 12.0$ ,  $52.4 \pm 9.7$  and  $56.4 \pm 8.5$  (no significant difference between groups) for the young healthy, elderly healthy and CHF groups respectively. Corresponding values for SNP were  $36.5 \pm 6.5$ ,  $42.8 \pm 6.5$  and  $99.7 \pm 37.9$  (no significant difference between groups).

### Plasma samples

The mean BNP level was approx. 20-fold higher in the CHF group compared with the age-matched controls (Table 2). In the CHF group, there were signs of a low-grade inflammation with elevated plasma levels of CRP, IL-6, sIL-2r and uric acid. The mean TNF $\alpha$  level



**Figure 3** Maximal increases in cutaneous blood flow (percentage of baseline) following local warming to +44 °C for 10 min in CHF patients, age-matched controls and young controls

CHF patients ( $n=15$ ), age-matched controls ( $n=14$ ) and young controls ( $n=6$ ). Values for the changes in cutaneous blood flow are expressed as a percentage of the baseline and are means  $\pm$  S.E.M. \* $P < 0.05$  Kruskal–Wallis/Mann–Whitney test.

was higher in patients, but the difference did not reach statistical significance. There was no marked change in IL-8 concentration, and the concentration of IL-1 $\beta$  did not reach detection level in any of the subjects studied. The changes in cytokine concentrations were relatively small, however, and only the sIL-2r level exceeded the range for normal variation set by the laboratory (Figure 4).

The vascular risk factor profile was changed in CHF patients with a higher (non-fasting) blood glucose

level ( $6.1 \pm 0.3$  compared with  $4.7 \pm 0.3$  mmol/l), plasma Hcy ( $17.6 \pm 1.2$  compared with  $13.3 \pm 0.6$   $\mu$ mol/l) and a lower LDL-cholesterol ( $2.7 \pm 0.13$  compared with  $3.8 \pm 0.18$  mmol/l), compared with the controls. High-density lipoprotein (HDL)-cholesterol was not affected.

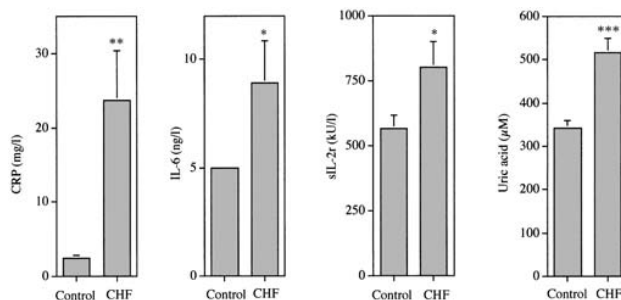
There was a higher vWf concentration in CHF patients ( $2.8 \pm 0.24$  compared with  $1.7 \pm 0.15$  international units/ml) indicating (prothrombotic) EDF. Also, the mean PAI-1 level was higher ( $7.7 \pm 2.7$  compared with  $4.0 \pm 1.0$  international units/ml), but the difference did not reach statistical significance.

### Correlations

The severity of CHF, measured as plasma BNP concentrations, was moderately correlated with several of the inflammatory markers, including IL-6, IL-8, CRP ( $r \geq 0.5$ ,  $P < 0.01$ ) and uric acid ( $r = 0.6$ ,  $P < 0.001$ ). There was also a fair-to-moderate correlation between BNP and some vascular risk factors:  $\beta$ -glucose, Hcy (both  $r = 0.4$ ,  $P < 0.05$ ) and LDL-cholesterol ( $r = -0.5$ ,  $P < 0.01$ ). The correlation with pulse rate was fair ( $r = 0.4$ ,  $P < 0.05$ ). There was no correlation with vascular reactivity, but a moderate correlation with plasma concentrations of vWf ( $r = 0.6$ ,  $P < 0.001$ ) and PAI-1 ( $r = 0.4$ ,  $P < 0.05$ ).

There was also fair-to-moderate correlation between inflammatory activity and the vascular risk factors. LDL-cholesterol was inversely correlated with both uric acid and IL-6 ( $r = -0.6$ ,  $P < 0.01$  and  $r = -0.4$ ,  $P < 0.05$  respectively). Hcy positively correlated with IL-6, sIL-2r and uric acid ( $r = 0.5$ ,  $P < 0.01$ ,  $r = 0.6$ ,  $P < 0.001$  and  $r = 0.5$ ,  $P < 0.01$  respectively) and negatively to basal vascular resistance and body mass index (BMI; both  $r = -0.5$ ,  $P < 0.05$ ).

The prothrombotic factors vWf and PAI-1 exhibited a fair correlation with each other ( $r = 0.4$ ,  $P < 0.05$ ). Several of the inflammatory markers co-varied with the vWf concentration (IL-6 and sIL-2r,  $r = 0.5$ ,  $P < 0.01$ ; and sCRP,  $r = 0.4$ ,  $P < 0.05$ ). The corresponding value for



**Figure 4** Plasma markers associated with inflammation in CHF patients and age-matched controls

\* $P < 0.05$ , \*\* $P < 0.01$  Mann–Whitney  $U$  test.

Hcy was  $r = 0.4$ ,  $P < 0.05$ ). sCRP also correlated with the PAI-I levels ( $r = 0.6$ ,  $P < 0.001$ ).

In the elderly groups, BMI correlated both the cutaneous basal vascular resistance and mean arterial pressure ( $r = 0.6$ ,  $P < 0.01$ ). There was no clear correlation between mean arterial pressure and vascular resistance however.

A few of the parameters co-varied with the blood flow responses. The AUC for the response to ACh and the maximal response to heat correlated negatively with the concentration of sIL-2r ( $r = -0.4$ ,  $P < 0.05$ ) and positively to LDL-cholesterol ( $r = 0.6$ ,  $P < 0.001$  and  $r = 0.4$ ,  $P < 0.05$  respectively). The AUC for both ACh and SNP was negatively correlated with vWF concentration ( $r = -0.4$ ,  $P < 0.05$ ). The corresponding values for SNP correlated negatively with the CRP level ( $r = -0.4$ ,  $P < 0.05$ ).

## DISCUSSION

### Blood flow measurements

To the best of our knowledge, this is the first time that a non-invasive method has been used for the study of vascular function in CHF patients. We correlated the blood flow responses in the cutaneous microvessels to inflammatory activity, aging and severity of CHF. The vasorelaxation following pharmacological stimulation and heat was greater in young adults than in elderly subjects; it can thus be concluded that the general vasodilatory capacity in the skin is reduced with advancing age. When the two elderly groups were compared, the responses to either vasodilator were lower in the CHF group. It seems likely that this may contribute to several of the patient's symptoms. The response to heat did not differ between the two elderly groups and its magnitude was higher than the response to SNP/ACh. This indicates that the maximal vasodilatory capacity was preserved in this group of CHF patients, but that there was a rightward shift in the dose-response curves for the pharmacological stimuli.

Both high age and the presence of CHF attenuated the responses to SNP and ACh to a similar extent. Thus our results do not support the hypothesis that the reduced vasorelaxing ability in CHF is secondary to EDF. Instead they suggest that the responsiveness of the contractile elements or the mechanisms directly regulating their tone was reduced. The responses to each of the pharmacological stimuli relative to the response of heat did not differ significantly for any of the groups, which gives further support to this view. It could still be, however, that the vasodilatory mechanisms of the endothelium are dysfunctional, but the changed action of the end organs makes this difficult to detect.

These results are consistent with the findings of Negrao et al. [8], who suggested that the attenuated resting or reflex-mediated vascular vasodilation in CHF was due to

heightened vasoconstrictor influences, such as increased sympathetic neural outflow, angiotensin and endothelin activity. The estimated values for basal vascular resistance at rest did not vary significantly in our population, however, indicating that, in this vascular bed, there is no significant vasoconstriction in any of the groups. Although our data should be interpreted with some caution, since the flow values are semi-quantitative and measurements were made on only a small fraction of the total cutaneous circulation, the results are supported by a similar finding in a study using the invasive forearm technique [16]. It thus raises some doubts as to whether the skin vessels in CHF patients are constricted at rest.

Another possible explanation for the reduced effect of SNP could be the presence of an abnormal end-organ response, i.e., a 'vascular stiffness' that makes blood vessels poorly responsive to vasodilator stimuli in general. One mechanism for this appears to be increased sodium content of the blood vessels [2]; however, other factors such as cellular stress and hypoxia can inhibit vascular relaxation in reactions involving heat shock proteins [17].

As mentioned above, several other reports in this area have, in contrast with our present observation, indicated the presence of EDF as the major cause for the impaired vasodilation. There are several possible explanations for the conflicting results. One is the difference in methodology. The method used in the present study is capable of detecting a selective reduction of endothelium-dependent vasodilation; this has been shown in several conditions such as children born with a low birth weight [18] and in heart transplant recipients [19]. In animal models of heart failure, however, it has been reported that endothelium-dependent relaxation is not uniformly impaired throughout the arterial bed [20]. The most commonly used method for this type of study [3,4,16] is the invasive forearm technique [11], which gives values for the net vascular resistance in the skin and muscle. The portion of cardiac output that reaches the skeletal muscle at rest is approx. twice as much as that which reaches the skin [21], and it could be that in CHF the vascular function in the skeletal muscle circulation differs from that of the skin. Another possible cause for the discrepancy could be differences in the study population. The patients in the present study were older than in most other studies and they were at an advanced stage of the disease. Bank et al. [16] observed in a study of CHF patients approx. 20 years younger than in our present study that EDF mainly influenced the blood flow in early stages of CHF syndrome but, similar to our results, the responses to both endothelium-dependent and -independent dilators were reduced at more advanced stages. Finally, pharmacological therapy could have affected the results. We did not attempt to discontinue any treatment, and it is known that angiotensin-converting-enzyme inhibitors improve endothelium-mediated vasodilation in patients with heart failure [22].

### CHF, inflammation and vascular reactivity

CHF was associated with an inflammatory reaction as indicated by the elevated levels of cytokines and sCRP. The increased levels of sIL-2r, which is a well-known marker for T-cell activation, suggests that the immune system could be one initiator of the response. The mean uric acid level was, as expected ([13,14], and references therein), elevated by over 50% in the CHF group and there was a weak, but significant, correlation between some inflammatory parameters (IL-6 and sIL-2r) and uric acid levels (results not shown). Both cytokines and the uric acid concentrations co-varied with the severity of CHF measured as plasma levels of BNP. These findings are in agreement with several other reports. For cytokines, one of the largest is a sub-study to the MERIT-HF trial [23], where increases in TNF $\alpha$  and IL-8 were also reported. The elevation of these cytokines did not reach statistical significance in our study, most probably due to the smaller study size.

Interestingly the T-cell activation marker sIL-2r correlated positively with the reduction in endothelium-dependent vasodilation. Besides this, we could not detect any significant co-variation between inflammatory activity and the blood flow responses. This was somewhat unexpected, since the post-ischaemic vascular response in the leg has been reported to correlate to uric acid levels [13] and inflammation can induce EDF (see Introduction). Furthermore, several of the possible mechanisms that may induce hyperuricaemia could, on theoretical grounds, be expected to influence vascular reactivity. In the peripheral vasculature and the heart, the endothelial cells are the predominant site of xanthine oxidase (XO), the enzyme involved in uric acid production [13]. This makes it possible that, from a structural point of view, XO could be affecting vascular function. XO activity is stimulated by tissue hypoxia and this parameter is likely to be directly linked to the vasodilatory capacity and to co-vary with the severity of CHF [24]. Also, the inflammatory response could be expected to induce EDF through an XO-mediated mechanism, since this enzyme contributes to ROS formation [13] and it can be induced by cytokines such as IL-6 and TNF $\alpha$  [25]. Thus we did expect a positive co-variation between uric acid levels and vascular reactivity, and our failure to detect this could possibly be due to a too small sample size or interference from factors that affect the uric acid levels, but do not affect the vascular function to a large extent. In our present study, the uric acid levels were reasonably correlated with serum creatinine (results not shown), and renal function could be one such parameter. Also, chronic diuretic therapy will raise uric acid concentrations [24].

### Vascular risk factors, CHF and blood flow

In the present study, we measured the levels of some well-known vascular risk factors that were expected to

influence vascular reactivity. The mean LDL-cholesterol level was significantly lower in CHF patients than in age-matched controls. Our blood samples were obtained from non-fasting subjects and the results may have been influenced by an uneven distribution of nutritional intake. The mean level in the CHF group was even below the reference value for the laboratory, which supports the interpretation that the detected difference was real and a part of the disease. It has been shown previously [26] that the cholesterol concentration has a biological relevance for CHF syndrome, since a low value is associated with poor clinical outcome. The positive correlation between LDL levels and vascular reactivity was unexpected, since hypercholesterolaemia is known to induce EDF [4]. It seems reasonable to suggest that some upstream factor(s) affects both the vascular function and the LDL concentration. LDL-cholesterol is known to be a negative acute-phase reactant [27] and thus be initiated by the inflammatory reaction. In agreement with this, we found that the LDL concentration was negatively correlated with IL-6. The acute-phase reaction is also reported [28] to be associated with a change in LDL particle composition into a more atherogenic type, and it could be that this has more pronounced effects on vascular function than the naive one.

Although patients with diabetes mellitus were excluded from the study, the mean blood glucose level was somewhat higher in the CHF group. This is likely to be due to an impaired glucose tolerance (IGT), which is a well-known characteristic of an inflammatory reaction or other forms of physical stress. IGT seems to be firmly associated with EDF [28], but we could not detect any correlation between blood glucose values and vascular reactivity. This could be due to the fact that it is less likely that a single non-fasting glucose value will give a good estimate of the degree of IGT.

The higher mean Hcy values in the CHF group and the positive correlation between Hcy and BNP concentrations indicate that hyperhomocyst(e)inaemia is part of CHF syndrome. It has been suggested [29] that Hcy can initiate a cascade of inflammatory pathways acting on the vascular cells; however, the relationship between the acute-phase response and Hcy seems to be complicated, and Hcy levels have been reported [30] to have an almost inverse relation to the acute-phase response after myocardial infarction. Hcy is reported to have pronounced vascular effects and acute elevation of the plasma concentration has been shown to induce EDF [31]. We observed that Hcy was associated with the inflammatory reaction with a positive correlation with sIL-2r and IL-6 levels. Also, other factors could be involved: the positive correlation with plasma creatinine suggests that an impaired renal function could have contributed, and the negative correlation with BMI could indicate that the nutritional intake influenced the result. Hcy concentration co-varied with the basal vascular

resistance, a finding of uncertain significance, but, besides this, we could not detect any correlation between the vascular reactivity and Hcy.

### Prothrombotic endothelial function

CHF is an independent risk factor for thromboembolic disease [10]. Part of this could be explained by rheological factors: blood that flows slowly tends to clot. In our present study group, we also found that the endothelium-derived factor vWF was elevated in the CHF group and that this correlated with several of the inflammatory markers as well as to the severity of CHF measured as BNP. The association between the acute-phase response and the vWf activity has been known for a number of years [32] and it seems likely that the prothrombotic activity is secondary to the inflammatory response. We could thus detect an EDF (prothrombotic) with a reasonable correlation with the impairment of vascular reactivity. The data are thus in line with the hypothesis by Becker et al. [9] in which the endothelium behaves in two principal modes, but we could not detect a specific reduction in the endothelium-dependent vasodilator response, possibly due to the attenuation of the vascular smooth muscle function.

### Conclusions

The present study shows that, with increasing age, reactivity in the cutaneous microvessels is attenuated. The presence of CHF causes further impairment, which, in our population, had a magnitude close to the effect of 50 years of aging. The reduction seemed to be caused by an impaired vascular smooth muscle function. CHF syndrome also induced an EDF as indicated by the elevated prothrombotic activity, but we could not with certainty detect a specific attenuation of endothelium-dependent vasodilation. A central part of CHF syndrome is a low-grade inflammation and this could induce secondary changes such as EDF, reduction in LDL-cholesterol, elevation of blood glucose and hyperuricaemia. The inflammation is associated with T-cell activation, and hyperhomocyst(e)inaemia may also be a possible contributing factor.

It can be concluded that it is possible to monitor microvascular function with this non-invasive technique. Further studies are required to determine the correlation between the changes in vascular reactivity, clinical symptoms and prognosis.

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# Paper III



## Reduction of Homocysteine in Elderly with Heart Failure Improved Vascular Function and Blood Pressure Control but did Not Affect Inflammatory Activity

Sven E. Andersson, Marie-Louise Edvinsson and Lars Edvinsson

Department of Internal Medicine, Lund University Hospital, S-226 85 Lund, Sweden

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**Abstract:** We have previously shown that hyperhomocysteinaemia is common in elderly heart failure patients, and is associated with endothelial dysfunction, impaired vasodilatory capacity and a low-grade inflammation. In the present study we examined if supplementation with B<sub>6</sub>, B<sub>12</sub> and folate could normalize the hyperhomocysteinaemia and if so, in turn, would improve the associated parameters. This was an open study without placebo control on heart failure patients with plasma homocysteine > 15 µM. Measurements of cutaneous vascular reactivity, blood pressure, inflammatory activity and endothelial function were performed before and after intervention with intra-individual comparisons. The treatment reduced homocysteine to near normal values and enhanced the hyperaemic response to acetylcholine related to the response to heat. The mean arterial blood pressure and pulse rate was reduced. There was no effect on inflammatory activity, plasma levels of von Willebrand factor, subjective health quality or the hyperaemic responses to sodium nitroprusside or local warming. Hyperhomocysteinaemia in heart failure patients is multifactorial in origin. Folate deficiency, inflammatory activity and reduced renal function could be contributing. It is suggested that supplementation with B-vitamins can improve the vasodilatory capacity and reduce the blood pressure but additional studies are required to confirm this.

There are several reports that heart failure is associated with a reduced vasodilatory capacity. This is suggested to be of importance for the progression of the heart failure syndrome and for the development of the patient's subjective symptoms. In a previous study on elderly heart failure patients we observed that the reactivity in cutaneous microvessels to both endothelium-dependent and -independent stimuli was attenuated (Andersson *et al.* 2003). This dysfunction seemed to be associated with a low grade inflammatory response. We also found that more than one third of the patients had clinical hyperhomocysteinaemia, defined as plasma homocysteine >12 µM (De Vriese *et al.* 2002). In accordance, the mean plasma homocysteine concentration was elevated in the heart failure group when compared to age- and sex-matched controls. The homocysteine level correlated positively with the severity of the heart failure, several inflammatory markers and to von Willebrand factor, a marker of prothrombotic endothelial dysfunction. It is not clear which mechanism induces the hyperhomocysteinaemia. It is possible that homocysteine is elevated as a part of an acute phase response. Such increases are described in the first phase both after stroke (Hovard *et al.* 2002) and myocardial infarction (Senaratne *et al.* 2000). It has been suggested that immune activation and the subsequent inflammatory response can induce a rise in homocysteine (Schroeksnadel *et al.* 2003). In agreement, homocysteine is increased in patients with inflammatory disease

such as rheumatoid arthritis, and is then related to markers of inflammation (Yxfeldt *et al.* 2003). One possible mechanism is that inflammation stimulates the degradation of vitamin B<sub>6</sub>, which leads to an increase in plasma homocysteine (McCarty 2000), but also effects on folate and cobalamines are suggested as well as direct production of Hcy in immune cells (Schroeksnadel *et al.* 2003).

There is an abundance of reports suggesting a causal relationship between elevated homocysteine and cardiovascular and thromboembolic disease (Wald *et al.* 2002). Several mechanistic pathways are proposed to be involved. For example, hyperhomocysteinaemia is reported to induce endothelial dysfunction and to impair vasodilatory capacity (Splaver *et al.* 2004). Homocysteine enhances vascular inflammation in animal models and augments the production of the pro-thrombotic tissue factor (Hofmann *et al.* 2001). This is possibly due to direct proinflammatory effects: Homocysteine increases production of hydroxyl radicals (Splaver *et al.* 2004), activates the transcription factor NF-κB and induces production of proinflammatory cytokines in monocytes (van Aken *et al.* 2000; Wang *et al.* 2001). Homocysteine could thus be induced as a part of an inflammatory response, but at the same time contributes to disease progression when exerting prooxidative effects by itself and amplifying oxidative stress (Schroeksnadel *et al.* 2003).

Based on this and our previous results we hypothesised that in heart failure high homocysteine levels contribute to the endothelial dysfunction, reduced vasodilatory capacity, inflammatory activity and, in turn, low health quality. The present study was conducted to investigate if vit-

Author for correspondence: Sven E. Andersson, Department of Internal Medicine, Lund University Hospital, S-226 85 Lund, Sweden (fax +46 46 2110908, e-mail sven.ea@telia.com).

amin supplementation, with doses used in ordinary health care, has the ability to normalise plasma homocysteine in elderly heart failure patients, and furthermore, if this affected the parameters mentioned above.

### Materials and Methods

**Ethics.** The study was conducted in accordance with the Declaration of Helsinki. The Ethics Committee of Lund University approved of the protocol (LU 516-02). Informed consent was obtained from all subjects.

**Participants.** Patients diagnosed with heart failure based on symptoms, clinical signs and an elevated plasma level of N-terminal pro-brain natriuretic peptide (NT-proBNP) and with plasma homocysteine  $>15 \mu\text{M}$  were included. Exclusion criteria were presence of tremor or cognitive impairment which could jeopardize the measurements. Fourteen patients were included, most of them out-patients in primary care. Two were females. Mean age was  $81 \pm 1$  years and body mass index  $26 \pm 1$ . After inclusion the participants were interviewed by a research nurse and asked to answer questions regarding their subjective health, tobacco and caffeine use, physical capacity and medication. They were also given a briefing on their pharmacotherapy.

One patient was an active smoker and one used oral tobacco. Seven were former smokers but most of them had quit more than twenty years ago. The severity of the heart failure was quantified in several ways: the mean estimated New York Heart Association functional class was  $2.8 \pm 0.2$  and mean estimated maximal walking distance 400 meters with a range from 10 to 1000 m. The plasma NT-proBNP level is given below. The patients were on average treated with nine different drugs (range one to sixteen). Cardiovascular pharmacotherapy is summarized in table 1. It was not changed during the treatment period except for a self-reported improvement in compliance (below).

**Measurements.** On the first visit plasma samples were taken for measurement of: interleukin-6 (IL-6), soluble interleukin2-receptor (sIL2r), C-reactive protein, (CRP; high sensitive method), cobalamines, folate, von Willebrand factor (antigen), HbA1c, creatinine and uric acid. Plasma samples were analysed at Department of Chemistry, Lund University Hospital. Creatinine clearance was calculated according to the Cockcroft-Gault formula:  $(140 - \text{age}) \times \text{body weight (kg)} \times \text{K/serum creatinine } (\mu\text{M})$ . K (constant) was 1.25 for men and 1.03 for women).

The cutaneous blood flow response to the endothelium-dependent vasodilator acetylcholine (ACh), the endothelium-independent dilator sodium nitroprusside (SNP) administered by iontophoresis, and to local warming was determined as in our former study. ACh

(2%) was administered five times using anodal current, and SNP four times using cathodal current. After this the vasodilatory response to local warming ( $44^\circ\text{C}$  for 10 min.) was determined (Andersson *et al.* 2003).

Treatment was then given for six weeks with a daily dose of 3 mg pyridoxine (vitamin B<sub>6</sub>), 0.8 mg folate and 0.5 mg cyanocobalamin (vitamin B<sub>12</sub>) (TrioBe<sup>®</sup>, Recip, Sweden). At the end of the treatment period all measurements were repeated.

**Data, statistical analysis.** Data are given as mean  $\pm$  S.E.M. Statistical analysis was performed by the Students two-tailed t-test for paired data. For several of the measurements we did not obtain data from the initial stimulations due to technical difficulties. We therefore do not present the area under the stimuli-response curve. Instead the maximal value (=the value after the last stimulation) is given. Since the baseline blood flow differed between the measurements (see below) the relative changes are presented. Calculations were performed using StatView 5.0, Berkeley, CA.

### Results

#### *Effect on homocysteine and vitamin concentrations.*

Mean plasma homocysteine was  $17.9 \pm 0.6 \mu\text{M}$  at inclusion. Vitamin supplementation significantly reduced this to  $13.8 \pm 1.1$  ( $P < 0.01$ ). The level decreased in all subjects except for one with end stage renal disease (calculated creatinine clearance = 13 ml/min.), in whom an increase was observed despite elevated plasma vitamin levels. It should be noted that only four patients reached the currently accepted upper limit for homocysteine of  $12 \mu\text{M}$  (De Vriese *et al.* 2002). In all participants there was a marked elevation of plasma folate indicating a good compliance to the study drug. The mean concentration increased from  $15.8 \pm 2.7 \text{ nM}$  at inclusion (range 6.4–37.0) to  $43.6 \pm 0.7$  ( $P < 0.001$ ). Four of the participants had subnormal folate levels ( $<10.0$ ) before treatment. The plasma concentration of cobalamines was  $537 \pm 91 \text{ pM}$  on first visit (range 183–1475). None had subnormal values. After treatment the level had increased to  $636 \pm 85$  ( $p < 0.05$ ). Our laboratory has no ability to analyse vitamin B<sub>6</sub>.

#### *Plasma samples.*

Before treatment the calculated creatinine clearance was  $49 \pm 5 \text{ ml/min.}$  corresponding to a moderate decrease in renal function. It was not affected by the treatment. Neither was the plasma levels of uric acid, HbA1c, soluble interleukin2 receptor (sIL2r), IL-6 or von Willebrand factor or the severity of heart failure, measured as plasma NT-proBNP, affected by the vitamin supplementation (table 2).

#### *General circulatory parameters.*

The mean arterial blood pressure and heart frequency was lower at the second measurement ( $90.2 \pm 3$  versus  $95.8 \pm 3$  and  $70 \pm 3$  versus  $75 \pm 3$  respectively; both  $P < 0.05$ ). The pulse pressure did not differ between measurements.

#### *Health quality assessment and pharmacological treatment.*

The vitamin supplementation did not change the patients' subjective health quality or walking distance. At inclusion 13 of the participants felt that they were well informed on

Table 1.

Cardiovascular pharmacotherapy taken by the study patients.

| Pharmacological treatment                | Number of participants |
|--|------------------------|
| Loop diuretics                           | 9                      |
| Aldosterone inhibitor                    | 5                      |
| Digitalis                                | 4                      |
| $\beta$ -adrenergic antagonists          | 4                      |
| Calcium channel blockers                 | 3                      |
| Angiotensin-converting-enzyme inhibitors | 4                      |
| Angiotensin receptor blockers            | 2                      |
| Anticoagulantia/thrombocyte inhibitor    | 13                     |
| Long acting nitroglycerin                | 2                      |
| Statins                                  | 4                      |
| Insulin                                  | 3                      |

Table 2.

Plasma concentrations of parameters related to heart failure and inflammation before and after vitamin treatment.

|                   | Before treatment | After treatment |
|-------------------|------------------|-----------------|
| NT-proBNP (ng/l)  | 2607±637         | 2474±469        |
| HbA1c (%)         | 5.7±0.4          | 5.3±0.2         |
| Uric acid (µM)    | 422±27           | 423±37          |
| sCRP (mg/l)       | 13.2±6.8         | 13.6±7.1        |
| IL-6 (ng/l)       | 6.1±1.5          | 4.9±0.8         |
| sIL-2r (kU/l)     | 856±163          | 728±73          |
| vWf (ag) (IU/ml)  | 2.04±0.18        | 1.95±0.20       |
| Creatinine (mg/l) | 140±34           | 143±37          |

the rationale for their pharmacological treatment. Five did regularly refrain from taking one or several of the prescribed drugs. This number was reduced to two at the follow-up visit. At the first visit nine patients complained of problems related to the pharmacological treatment. The number was reduced to five at the follow-up. More than half of the group had technical help for correct dosing.

#### Vasodilatory responses.

The basal blood flow (before stimulation) differed between measurements. For ACh the value was 20.0±6.7 flow units at the first visit and 7.2±1.4 at the second ( $P<0.01$ ). Corresponding values were 7.25±1.4 and 19.8±6.8 (n s) for SNP and 17.4±3.5 and 11.6±1.6 ( $P<0.05$ ) for heat stimulation, respectively. The differences were obviously due to random variation. Stimulation with ACh induced an increase in blood flow of 578±110% at first visit and 782±105 at the second (n s;  $P=0.07$ ). For SNP the corresponding values were 394±99% and 524±92 (ns). Local warming induced a blood flow increase of 721±114% before treatment and 564±76 after (n s). There was a significant enhancement of the response to ACh when it was expressed as relative (per cent) to the response to heat (fig. 1). The response to SNP, calculated the same way, did not reach statistical significance ( $P=0.056$ ). These responses did not correlate to homocysteine levels, however.

#### Discussion

The present study was conducted in order to further investigate previous findings related to homocysteine (Andersson *et al.* 2003). The two study populations were similar with regard to NYHA functional class, BMI, MAP, Hcy concentration and medication heart failure. The present group was older, however, and had a more reduced renal function. No side-effects of the vitamin treatment were reported from the participants.

Our results show that vitamin supplementation can reduce the elevated homocysteine levels in heart failure patients. The treatment did not induce complete normalisation of homocysteine, however, and it can be speculated that the doses used were suboptimal. We can only speculate to which extent each of the three vitamins contributed to the result. A number of participants in the present study

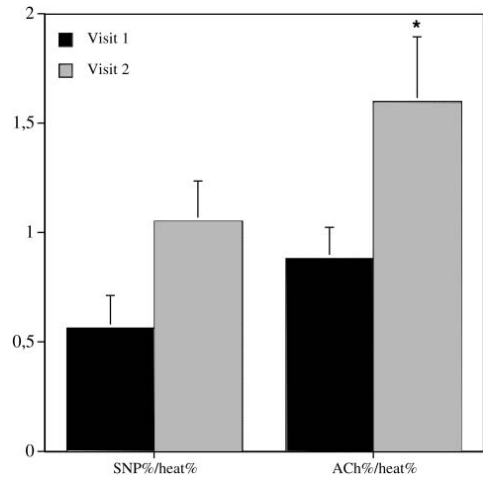


Fig. 1. The cutaneous vasodilatory responses to SNP and ACh (maximal response expressed as per cent of basal blood flow) expressed as per cent of the response to local warming; before and after vitamin supplementation. \*= $P<0.05$ .

had subnormal plasma folate concentrations although the mean plasma level was within the normal range. Folate deficiency could thus be one explanation for the elevated homocysteine. A similar conclusion was drawn from an earlier study with a larger group of psychogeriatric patients. Those patients were comparable in age to our group but had even higher homocysteine levels (Nilsson *et al.* 1999). The two groups also differed in that none of the participants in our study had subnormal levels of cobalamines before treatment whereas that was common in the psychogeriatric group. Our group thus had a higher mean plasma level of cobalamines. It might thus be that the mechanisms leading to hyperhomocysteinaemia differ between conditions and that cobalamine deficiency is of less importance in heart failure. We could not measure vitamin B<sub>6</sub> but the present data together with the correlation between inflammation and homocysteine seen previously fit in with the hypothesis mentioned earlier of a reduced availability of pyridoxine phosphate (McCarty 2000).

The study participants in general had mild to moderate renal failure. This is a well-known determinant of the homocysteine level (De Vriese *et al.* 2002 and references therein). The reduction in homocysteine did not affect the creatinine values. The one patient with end-stage renal disease exhibited increased homocysteine level despite elevated vitamin concentrations. This is in accordance with previous findings and is probably explained by the fact that the hyperhomocysteinaemia in patients with renal failure is to a large extent due to a marked reduction of homocysteine clearance from plasma. Folate does not affect the plasma elimination of homocysteine but enhances the remethylation in the tissues, which leads to a lower influx into

the plasma compartment. In our group of patients this effect might be sufficient to override the defect in elimination and obtain new lower steady state levels. This is not possible in end-stage renal disease, however (De Vriese *et al.* 2002). Taken together it could be that several factors contribute to the hyperhomocysteinaemia; renal dysfunction, inflammatory activity and vitamin deficiency being among these.

Despite the reduction in plasma homocysteine the measured markers of inflammatory activity remained constant. The data does thus not support the hypothesis that high homocysteine augments the inflammatory response in heart failure. The co-variation between inflammatory parameters and homocysteine seen in our former study is then rather explained by the opposite relationship; that inflammation raises homocysteine. Neither did the treatment reduce the levels of von Willebrand factor. This was a somewhat unexpected finding; it has previously been reported that an acute hyperhomocysteinaemia induced by methionine loading increases von Willebrand factor levels significantly (Tam *et al.* 2003). Our results rather suggest that, in heart failure, the relationship between this marker of endothelial dysfunction and homocysteine are influenced by a common upstream event but do not directly affect each other. In accordance, it has previously been reported that von Willebrand factor is increased in human beings with high homocysteine but the association is, at most, weak (Becker *et al.* 2000).

The participants could not detect any marked improvement in their subjective health quality. It should be kept in mind that they were old and most of them had multiple chronic diseases. It thus seems likely that minor or moderate improvements in physical abilities could pass unnoticed and that this parameter thus has a low sensitivity. Interestingly the opportunity to discuss the pharmacotherapy markedly improved the participants' concordance and reduced the discomforts they felt were related to medication. The blood pressure was significantly lower at the second visit. Since this study was open and without placebo control it is not possible to conclude if this was due to the increased adherence to the prescribed pharmacological treatment or to an unspecific effect. There are, however, previous reports that treatment with pyridoxine plus folic acid had a reducing effect on the blood pressure (van Dijk *et al.* 2001) and in a recent study it was shown that higher folate intake is associated with reduced risk of incident hypertension (Forman *et al.* 2005). It might thus be hypothesised that vitamin supplementation has a direct blood pressure-reducing effect in heart failure patients with hyperhomocysteinaemia, possibly mediated by an improvement of vascular function.

In several previous studies vitamin supplementation has been shown to improve vasodilator capacity concomitant with a reduction in homocysteine (Splaver *et al.* 2004). Some controversy remains whether this improvement is directly linked to the homocysteine reduction or if it is a parallel phenomenon (De Vriese *et al.* 2002; Stanger *et al.* 2002). The reports are unequivocal; vitamin supplementation seems not to have any beneficial effect on vasorelaxation when homocysteine is elevated secondary to renal fail-

ure (De Vriese *et al.* 2002). Similarly, it has recently been reported that supplementation with B-vitamins and folate does not improve flow-mediated vasodilation in older adults with mild hyperhomocysteinaemia (Carlsson *et al.* 2004). In our study the mean vasodilatory response to ACh and SNP was higher at the second visit but these changes did not reach statistical significance. The ACh-induced hyperaemia was significantly elevated, however, when related to the heat response. This suggests that the high homocysteine concentration contributes to the impaired vasodilatory capacity in heart failure patients but also the improved compliance to prescribed medication could have influenced the results. Additional studies are thus required to establish the link between homocysteine and vascular function in this condition and perhaps for a longer time period.

In conclusion, the present data suggests that the hyperhomocysteinaemia seen in heart failure is multifactorial in origin. Folate deficiency, inflammatory activity and reduced renal function are likely to contribute to the elevation, among others. Vitamin supplementation reduces plasma homocysteine in this condition and it is suggested that this treatment could improve the endothelium-dependent vasodilatory capacity and reduce blood pressure. We found no evidence of an improvement of the prothrombotic endothelial function.

#### Study limitations.

The small number of participants and the open-label design limit the study. Furthermore, the variations in the base-level flow could have influenced the vasodilatory results. These data could also have been affected by the increased compliance to the prescribed drugs.

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# Paper IV





## Deteriorated function of cutaneous microcirculation in chronic congestive heart failure

Marie-Louise Edvinsson, Erik Uddman, Sven E Andersson

Department of Emergency Medicine, Lund University Hospital, SE- 221 85 LUND, Sweden

### Abstract

**Background** Chronic congestive heart failure is a complex condition that leads to dysfunction in the peripheral microcirculation. We have previously shown that vascular reactivity is reduced with increasing age. In this study, we examined a group of very old patients with severe chronic heart failure to test the hypothesis that vascular function is further compromised by a combination of heart failure and aging. **Methods** Cutaneous forearm blood flow was measured by laser Doppler flowmetry and compared among three groups: Group 1 ( $n = 20$ , mean  $\pm$  SE:  $85.5 \pm 4$  years), heart failure patients with New York Heart Association class IV (NYHA IV) and with a NT-proBNP level  $\geq 5000$  ng/L; Group 2 ( $n = 15$ , mean  $\pm$  SE:  $76.5 \pm 2$  years), heart failure patients with NYHA II and NT-proBNP  $\leq 2000$  ng/L, and Group 3 ( $n = 10$ , mean  $\pm$  SE:  $67.6 \pm 3.0$  years), healthy controls with no clinical signs of heart failure. The vasodilator response to the iontophoretic administration of acetylcholine (ACh), acting via an endothelial mechanism, and sodium nitroprusside (SNP), acting via a smooth muscle cell mechanism, were studied. **Results** All patients with heart failure had significantly reduced vascular reactivity independent of the mode of stimulation (ACh, SNP or heat) when compared to healthy controls. However, the responses did not differ between the two groups of heart failure patients. **Conclusions** Cutaneous vascular reactivity is reduced in heart failure patients and does not correlate with the severity of the condition or age of patients.

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**Keywords:** heart failure; cutaneous microcirculation; endothelial responses; acetylcholine; smooth muscle responses

## 1 Introduction

Congestive heart failure is a multi symptomatic disease that is more abundant in the elderly population.<sup>[1]</sup> Patients with chronic heart failure are characterized by multi organ dysfunction and frequently seek hospital care because of shortness of breath and peripheral oedema. One of the features of heart failure is a low grade inflammation of unknown origin that is associated with reduced vascular responses to vasodilator stimuli.<sup>[2]</sup> It has been proposed that this vascular dysfunction could contribute to symptoms like fatigue and intolerance to heat.<sup>[3]</sup>

The vast majority of clinical research on heart failure has focused on middle aged (40–65 years) and the younger elderly (65–75 years) however. In previous studies, we have shown that cutaneous microvascular function declines in older patients and also that the endothelium-dependent

vascular reactivity appears to negatively correlate to the severity of heart failure.<sup>[2]</sup>

In this study, we aimed to investigate these phenomena by studying if age and severity of heart failure could have a synergistic effect on the microvasculature; or if the vascular dysfunction mainly is an early response in the heart failure syndrome.

## 2 Methods

### 2.1 Patients

The study population consisted of three groups. Group 1 consisted of 20 patients, 12 men and 8 women, mean age of 85.5 years. The patients were diagnosed earlier with chronic congestive heart failure. The heart failure patients arrived due to worsening of the condition to Lund University Hospital (Lund University, Sweden) with New York Heart Association class IV (NYHA IV) symptoms and NT pro-BNP levels  $\geq 5000$  ng/L. Group 2 consisted of 15 heart failure patients, 9 men and 6 women, who were obtained from the out patients clinic in the same geographic region, with mean age of 76.5 years. They were considered clinically stable with NYHA II symptoms and NT pro-BNP levels of about 2000 ng/L. Group 3 consisted of 10 healthy

**Correspondence to:** Marie-Louise Edvinsson, BSc, Department of Emergency Medicine, Lund University Hospital, SE-221 85 LUND, Sweden. Email: marie-louise.edvinsson@skane.se

**Telephone:** +46-46-171484

**Fax:** +46-46-2110908

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elderly age- and gender-matched subjects recruited from the community registry. These subjects had a mean age of 67.5 years. Their NT pro-BNP levels were in the normal range, 50–300 ng/L. They did not take any medication for cardiovascular disease.

The two groups of chronic congestive heart failure patients had reduced left ventricular function upon echocardiography and were all non-current smokers when entering the clinical study to avoid any effects on flow measurements.<sup>[4]</sup> Healthy controls have ejection fraction > 50%.<sup>[5]</sup> All patients were kept on their prescribed medication but refrained from long lasting nitrates six hours before the laser Doppler blood flow measurement. No other co-morbidity resulted in exclusion of participation in the study, only tremor was considered not suitable for the laser Doppler blood flow method. For demographic details on the subjects, see Table 1.

**Table 1.** The demographics of severe and moderate congestive heart failure patients vs. healthy subjects. Data are given as mean  $\pm$  SE, and/or range in parenthesis.

|                        | NYHA IV<br><i>n</i> = 20   | NYHA II<br><i>n</i> = 15  | Healthy<br><i>n</i> = 10  |
|------------------------|----------------------------|---------------------------|---------------------------|
| Age                    | 85.5 $\pm$ 1.2<br>(78–96)* | 76.5 $\pm$ 1.9<br>(68–84) | 67.6 $\pm$ 3.0<br>(56–81) |
| Sex, F/M               | 8/12                       | 5/10                      | 6/4                       |
| BMI, kg/m <sup>2</sup> | 24.6 (19–34)               | 26.4 (20–34)              | 28.4 (24–35)              |
| MABP                   | 94 $\pm$ 3.6               | 101 $\pm$ 3.2             | 90 $\pm$ 3.0              |
| Pulse/min              | 74 (60–90)                 | 74 (52–110)               | 70 (56–86)                |

NYHA: New York Heart Association classification; BMI: Body mass index; MABP: Mean arterial blood pressure. \**P* < 0.05 NYHA IV vs. NYHA II.

The investigation conformed to the principles outlined in the Declaration of Helsinki (Seoul 2008). The Ethics Committee of Lund University approved of the protocol (LU 465-03). Written informed consent was obtained from all patients by the investigator before they were entered into the study.

## 2.2 Clinical parameters

Hemodynamic measurements consisted of arterial blood pressure and heart rate. Blood pressure was measured non-invasively in the supine position from the upper left arm with the cuff inflated at heart level. Blood pressure was taken after the blood flow measurement when the patients had been resting for about one hour. The diastolic value was accepted as Korotkoff's phase V. All blood pressure measurements were taken by the same investigator. Heart rate was counted for one minute.

## 2.3 Blood analysis

Plasma levels of inflammatory markers, C-reactive protein (P-CRP), interleukin 6 (IL-6) and soluble IL 2 receptor

(s-IL2r) were measured as well as pro-brain natriuretic peptide (NT-proBNP), P-LDL (low density lipoprotein) cholesterol, P-HLD (high density lipoprotein) cholesterol, and blood glucose levels. In addition, haemoglobin (Hb), P-sodium, P-potassium, P-creatinine and P-uric acid were analyzed at the Department of Clinical Chemistry and Pharmacology. Interleukins were measured at Clinical Immunology laboratory at Lund University Hospital. All blood samples were obtained from a peripheral venous access in heart failure patients and measured by validated techniques.

## 2.4 Blood flow measurements

Cutaneous blood flow was measured using the PeriFlux system 5000 (Perimed, Järfälla, Sweden). This method is non-invasive and gives minimal discomfort to the patients which makes it suitable for severely ill patients at bedside.<sup>[6]</sup> Laser-generated light at a wavelength of 780 nm is directed to the skin using a fibre optic probe. The light reflected from moving blood cells in the superficial skin microvessels undergoes a shift in frequency (Doppler effect) that is proportional to the number and velocity of moving blood cells. The laser-Doppler output is semi-quantitative, and we have presented all data as the percentage change compared with the baseline perfusion value. Temperature of the skin was recorded continuously.

## 2.5 Iontophoresis

Constant current iontophoresis was used to enhance the perfusion of charged molecules into the skin of the dorsal side of the lower arm. Endothelium-dependent vasodilatation was provoked by iontophoresis of the acetylcholine (Ach, 2% dissolved in MilliQ water, Sigma) using anodal current to deliver the positively charged molecule.

Endothelium-independent vasodilatation was provoked by iontophoresis of nitric oxide (NO) donor, sodium nitroprusside (SNP, 1% dissolved in MilliQ water, Sigma) using the cathode current for this negatively charged molecule.<sup>[2]</sup> The PeriIont System (Perimed) used in this study contains of an applicator with a small recess in the centre and of circular temperature probe surrounding the application site. The recess in the centre allows the insertion of a fibre optic probe to measure the blood flow in the stimulated area. An additional temperature probe containing a fibre optic probe was placed at a distance suitable to avoid large veins. This was used as a reference during the iontophoresis and was subsequently used to determine the response to local warming.

All studies were performed at room temperature (22°C–24°C). For the severely ill congestive heart failure (CHF)

patients, the measurements were obtained at the hospital emergency ward. For both the CHF patient group from the out patient clinic and the healthy subjects, blood flow measurements were carried out at the Clinical Trial Centre, Lund University Hospital, Lund, Sweden. All subjects were resting in a supine position. Blood pressure and heart rate were measured before and after stimulation and the lowest value is given. The skin of the dorsal lower arm was gently cleansed and the iontophoretic applicators/fibre optic probes were applied to the forearm resting on a pillow to give comfort and provide stabilization. The basal blood flow was studied for 2 min after which ACh was transferred by iontophoresis (anodal current, 0.2 mA for 20 s). The current alone did not affect the blood flow (results not shown). The protocol was based on our previous study<sup>[2]</sup> when we determined that successive iontophoretic stimuli at 60 s intervals, produces a cumulative stimuli-response curve. We measured the maximum response after five stimuli. Endothelium-independent vasodilatation was studied by iontophoresis of SNP as above (cathode current, 0.1 mA for 60 s). The stimulation was repeated four times at 60 s intervals. Finally, the response to heat was measured following local warming to +44°C for 10 min. This responses were considered as maximum vasodilatation in the micro vessels of the skin using this technique.

## 2.6 Laser Doppler calculation

Light is transmitted to the tissue via a fibre-optic probe. When the light hits moving blood cells, it undergoes a change in wavelength (Doppler shift). The magnitude and frequency distribution of these changes are directly related to the number and velocity of blood cells, i.e., the blood perfusion. Measurements are expressed in arbitrary Perfusion Units (PU). Full linear correlation to absolute perfusion value is achieved using perimed's analysis technology (including a linearization function to avoid underestimation in highly perfused tissues) and calibration using automatic instrument zeroing and Perimed's Motility Standard.

The responses are expressed as the maximum percent change in PU from baseline flow (set as 100% in each subject) to the iontophoretic administration of ACh and SNP. The perfusion change after local heating (e.g., +44°C) is a measure of the tissue reserve capacity.

## 2.7 Statistical analysis

Statistical analysis was performed by Mann-Whiney *U* test. Statistical differences with a *P* value < 0.05 were considered significant. Calculations were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

## 3 Results

### 3.1 Basic characteristics

There was no significant difference between the two groups of heart failure patients regarding gender, body mass index (BMI) and blood pressure; however, the NYHA IV patients were older (Table 1). The concomitant diseases and the pharmacological treatment in the heart failure groups are given in Table 2. The difference in ejection fraction (EF) between the groups was expected due to the difference in severity of the heart failure. EF was 43% ± 3% in NYHA II and 34% ± 2 % in NYHA IV (*P* < 0.05 between the two heart failure groups). Blood samples also showed much higher levels of proBNP in NYHA IV (16859 ± 1966 ng/L) as compared to NYHA II (1959 ± 569 ng/L; *P* < 0.05). The healthy individuals had proBNP levels that were lower than 500 ng/L.

**Table 2.** Medical history and treatment of the chronic congestive heart failure patients.

|                               | CHF patients in hospital (n = 20) | CHF from out patient clinic (n = 15) |
|-------------------------------|-----------------------------------|--------------------------------------|
| NYHA II                       | 0                                 | 15                                   |
| NYHA IV                       | 20                                | 0                                    |
| Co-existing disease           |                                   |                                      |
| Hypertension                  | 2/20                              | 2/15                                 |
| Diabetes                      | 4/20                              | 3/15                                 |
| Coronary artery disease       |                                   |                                      |
| Prior myocardial infarction   | 8/20                              | 4/15                                 |
| Electrocardiogram             |                                   |                                      |
| Arterial fibrillation         | 9/20                              | 7/15                                 |
| Bundle branch block           | 6/20                              | 0/15                                 |
| Pacemaker                     | 4/20                              | 1/15                                 |
| Chest X-ray                   |                                   |                                      |
| Pulmonary oedema              | 12/20                             | 0/15                                 |
| Cardiomegaly                  | 16/20                             | 0/15                                 |
| Pharmacological treatment     |                                   |                                      |
| Beta-adrenoceptor antagonists | 13/20                             | 8/15                                 |
| ACE-inhibitors                | 12/20                             | 12/15                                |
| Digoxin                       | 1/20                              | 2/15                                 |
| ARB                           | 1/20                              | 0/15                                 |
| Diuretics                     | 20/20                             | 6/15                                 |
| ASA                           | 12/20                             | 6/15                                 |
| Warfarin                      | 4/20                              | 6/15                                 |
| Spironolactone                | 4/20                              | 2/15                                 |

CHF: congestive heart failure; *n*: number of patients; NYHA: New York Heart Association classification; ACE: angiotensin converting enzyme; ASA: acetylsalicylic acid; ARB: angiotensin receptor blockers.

Other clinical parameters that differed between the groups were significantly lower Hb and HDL and higher CRP, creatinine, uric acid, IL-6 and s-IL2r in NYHA IV as compared to NYHA II. The calculated absolute glomerular filtration rate<sup>[7]</sup> was  $64 \pm 4$  mL/min in NYHA II and  $35 \pm 5$  mL/min in NYHA IV ( $P < 0.0001$ ).

The above differences are consistent with a more severe disease state in NYHA IV patients as compared to the NYHA II group and provide clear indication of ongoing inflammation in the former group. Six months after participation in this study, 12 out of 20 subjects had died in the NYHA IV group while only one patient had died in the NYHA II group. These findings agree with a previous mortality study of severe heart failure.<sup>[8]</sup>

### 3.2 Microvascular responses

ACh stimulates the release of NO and elicits a subsequent dilatation of cutaneous blood vessels. The control subjects showed a mean dilatation of  $975 \pm 120\%$ , relative to baseline resting flow (which was set as 100%), the NYHA II patients showed a mean dilatation to ACh of  $659 \pm 124\%$  ( $P < 0.05$  vs. healthy controls) while the severe heart failure subjects (NYHA IV) had a mean dilatation of  $588 \pm 127\%$  ( $P < 0.005$  vs. healthy controls).

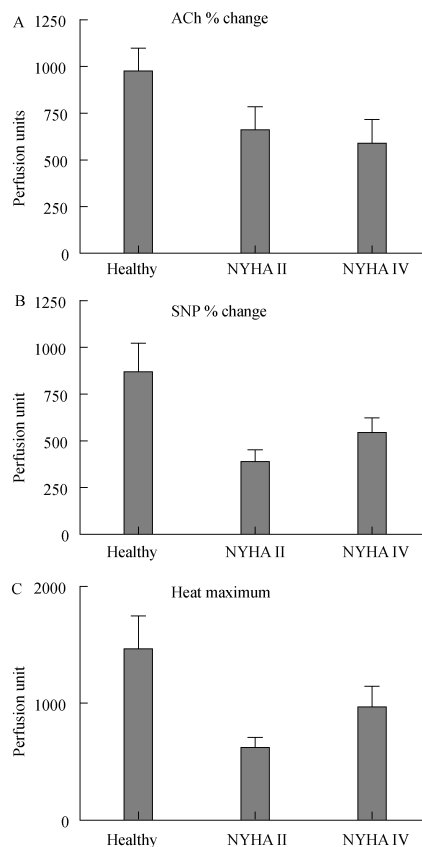
SNP mediates its effect directly on the smooth muscle cells independent of the endothelium.

The responses to SNP in the controls were more variable than responses to ACh (Figure 1). The mean value was  $869 \pm 153\%$  in the healthy controls. There was a markedly lower response to SNP in the NYHA II group ( $389 \pm 63\%$ ;  $P < 0.05$ ) as compared to controls. A reduction in responsiveness were seen also in the severe heart failure group ( $544 \pm 79\%$ ;  $P < 0.005$  as compared to controls).

Heat causes a non-endothelium dependent dilatation of the vascular bed and is used as a way to cause near maximum dilation of the local cutaneous vascular bed. We found that the response to heat was  $1467 \pm 283\%$  in the healthy controls, and this was markedly reduced in both mild ( $621 \pm 87\%$ ;  $P < 0.01$ ) as compared to healthy controls and severe heart failure ( $969 \pm 177\%$ ;  $P < 0.05$ ) as compared to healthy controls.

## 4 Discussion

The present study has shown that patients with CHF have reduced microvascular reactivity to acetylcholine, nitroprusside and local heat. In a previous study, we found that the microvascular relaxant capacity in skin vessels was reduced in healthy elderly people and attenuated further by presence of heart failure.<sup>[2]</sup> However, contrary to our expectations,



**Figure 1.** Percent increase in blood flow (perfusion units) compared to base line (set as 100%) in healthy individuals ( $n = 10$ ), patients with congestive heart failure of NYHA II ( $n = 15$ ) and NYHA IV ( $n = 20$ ). (A) endothelium-dependent responses to acetylcholine; (B) endothelium-independent relaxation response to sodium nitroprusside; (C) general vasodilator response to local heating to  $+44$  °C. Values represent mean maximum relaxation  $\pm$  SE. NYHA: New York Heart Association classification; ACh: Acetylcholine; SNP: sodium nitroprusside.

there was no significant correlation between the severity of heart failure and cutaneous microvascular dysfunction. Even though they were elderly, the patients with more severe symptoms (NYHA IV) showed similar vasodilatory responses as less severely ill patients (NYHA II).

In the present study, we explored the relation between these parameters (age, heart failure) by measuring the vascular responses in a group of elder elderly patients with

heart failure at the terminal stage. Unexpectedly, the vascular responses did not differ markedly between the two heart failure groups. Our previous studies had shown that vasomotor reactivity is diminished during transition from youth to middle-aged due to normal aging processes.<sup>[2]</sup> Heart failure further decreases vasodilatory responses in elderly patients of 65–70 years. However, in our data from very old heart failure patients (78–96 years), there was no further age related decline and, the blood vessels ability to relax appeared stable.

It is remarkable that our group of very old patients who were seriously ill and had all indications of ongoing elevated inflammation had similar vasodilatory responses as the group of milder heart failure patients. This suggests that microvascular insufficiency is not worsening with the progression of severe chronic congestive heart failure.

This study has shown that subjects with CHF have reduced vascular capacity as assessed using local application of heat (+44°C) as compared to matched elderly control subjects. Similarly the iontophoretic administration of ACh and SNP resulted in significantly lower dilator responses as compared to the healthy group; this difference was somewhat larger for the dilatation to SNP. It was obvious that the two heart failure patients groups had markedly lower cutaneous vasoreactivity as compared to the healthy controls. There was no stepwise reduction in cutaneous responses that depended on the severity of heart failure.

The reason behind this is not clear, but we can exclude some factors based on our choice of the design: (1) Age and gender were not confounders because the subjects were matched to a similar degree as we have analysed before.<sup>[2]</sup> (2) Smoking may severely affect the cutaneous responses, however we excluded such subjects in this study.<sup>[4]</sup> (3) Degree of inflammation in the vasculature may be a confounder and we have earlier published that homocysteine, CRP and cytokines could be a marker of heart failure.<sup>[9]</sup> The present subjects had several markers of inflammation that differed between the two groups of heart failure (Table 3). Thus, higher levels of CRP, IL6 and IL2r were seen in NYHA IV patients as compared to NYHA II. Interestingly, both heart failure groups had attenuated responses to ACh, SNP and heat as compared to that seen in the healthy controls. If anything, the reductions were more pronounced in the mild NYHA II group as compared to the severe heart failure group which may argue against a purely causative role of inflammation in diminished vasorelaxant reactivity.<sup>[9]</sup> and (4) Other clinical parameters such as plasma sodium and potassium were within acceptable values and can thus not be confounders. The levels of NT-proBNP were typical for the two stages of heart failure: > 2000 ng/L vs. > 16000 ng/L,

**Table 3.** Laboratory blood analysis, mean ± SE.

|                     | CHF NYHA II out patients<br><i>n</i> = 15 | CHF NYHA IV hospitalized<br><i>n</i> = 20 |
|---------------------|---|---|
| NT pro BNP (ng/L)   | 1959 ± 569                                | 16859 ± 1966*                             |
| Hemoglobin (g/L)    | 143 ± 3.7                                 | 116.5 ± 4.8*                              |
| Sodium (mmol/L)     | 141 ± 0.9                                 | 141 ± 1.1                                 |
| Potassium (mmol/L)  | 4.2 ± 0.1                                 | 3.9 ± 0.1                                 |
| Creatinine (µmol/L) | 96.8 ± 5.4                                | 152.7 ± 16.0*                             |
| Uric acid (µmol/L)  | 450 ± 21                                  | 559 ± 36*                                 |
| LDL (mmol/L)        | 3.1 ± 0.2                                 | 2.1 ± 0.2                                 |
| HDL (mmol/L)        | 1.8 ± 0.2                                 | 1.1 ± 0.1*                                |
| CRP (mg/L)          | 4.7 ± 0.7                                 | 13.3 ± 4.6*                               |
| HbA1c (%)           | 4.8 ± 0.1                                 | 7.0 ± 0.3*                                |
| IL-6 (ng/L)         | 5.8 ± 2.1                                 | 7.0 ± 0.3*                                |
| IL-2r (kU/L)        | 666 ± 88                                  | 1127 ± 123*                               |

CHF: Congestive heart failure; NYHA: New York Heart Association classification; NT pro BNP: Nerve terminal-pro-brain natriuretic peptide; LDL: Low density lipoprotein; HDL: High density lipoprotein; CRP: Sensitive C Reactive Protein; IL: Interleukin; IL-2r: Soluble IL 2 receptor.  
\**P* < 0.05 compared between the two groups.

and are somewhat in accordance with the ejection fraction measurements obtained in conjunction with echocardiography (43% and 36%, respectively). We suggest that proBNP is a better predictor of severity of heart failure as compared to echocardiography in the standard clinical setting.<sup>[8]</sup> Since the microvascular response was not different for the moderate and severe heart failure patients groups, it may indicate that the cutaneous vascular reactivity to vasodilators is an early sign in CHF and reveals alteration of the circulatory system to congestion.

One consideration is that the age of the NYHA IV patients was beyond the average life span. Thus, patients in this group may reflect a selected group who, due to genetics, lifestyle, diet and other factors, may have been better able to preserve endothelial function as compared to the general population of heart failure patients.

In conclusion, patients with heart failure have reduced microvascular responses to endothelial and smooth muscle cell stimulants. However, there was no relation to the degree of chronic heart failure which may suggest that the vascular reduction in responses may be an early sign, but not a progression indicator of chronic heart failure.

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# Paper V



**Brain natriuretic peptide is a potent vasodilator in human microcirculation but the response is down regulated in heart failure patients.**

**Marie-Louise Edvinsson, Erik Uddman, Lars Edvinsson, Sven E. Andersson**

Department of Emergency and Internal Medicine, Institute of Clinical Sciences Lund University, Lund University Hospital, Lund, Sweden.

Correspondence:

Lars Edvinsson

Department of Emergency Medicine,

Lund University Hospital, Lund University,

SE- 221 85 LUND, SWEDEN

E-mail: [lars.edvinsson@med.lu.se](mailto:lars.edvinsson@med.lu.se)

*Abstract:*

**Aims** Brain natriuretic peptide (BNP) is normally present in low levels in the circulation, but it is elevated in parallel with the degree of congestion in heart failure subjects (CHF). BNP has natriuretic effects and is a potent vasodilator. It is suggested that BNP could be a therapeutic alternative in CHF. However, we postulated that the high levels of circulating BNP in CHF may downregulate the response of microvascular natriuretic receptors. This was tested by comparing 15 CHF patients (BNP>3000) with 10 matched, healthy controls.

**Methods and results** Cutaneous microvascular blood flow in the forearm was measured by laser Doppler flowmetry. Local heating (+44°C, 10 min) was used to evoke a maximum local dilator response. Non-invasive iontophoretic administration of either BNP or acetylcholine (ACh), a known endothelium-dependent dilator, elicited an increase in local flow. The nitric oxide synthase inhibitor L-NAME blocked the BNP response, indicating that BNP acts via nitric oxide. Interestingly, responses to BNP in CHF patients were reduced to about one third of those seen in healthy controls (increase in flow: 251% in CHF vs. 908% in controls;  $p < 0.001$ ). The increase in absolute perfusion units also showed robust reduction to the BNP administration between the two patient groups ( $p < 0.05$ ). In contrast, the vasodilator responses to ACh and to local heating were only somewhat attenuated in CHF patients. Thus, dilator capacity and nitric oxide signalling were not affected to the same extent as BNP-mediated dilation, indicating downregulation of the latter response.

**Conclusions** The findings show for the first time that microvascular responses to BNP are markedly reduced in CHF patients as compared to age-matched controls. This is consistent with the hypothesis of BNP receptor function is downregulation in CHF.

**Key words:** heart failure, cutaneous microcirculation, endothelial responses, acetylcholine, brain natriuretic peptide, nitric oxide.

## **Introduction**

Patients with congestive heart failure exhibit a number of cardiac conditions both structural and functional such as left-ventricular systolic and diastolic dysfunction, abnormal right-ventricular size and function, valvular heart disease, pulmonary artery hypertension and arterial fibrillations [1]. The outcome of acute heart failure is still dismal with high rates of early death and rehospitalisation and there is no major improvement over the past decades [2]. Myocyte stress from volume overload and myocardium wall stretch triggers natriuretic peptide gene expression and the release of atrial and brain natriuretic peptides (ANP/BNP) and NT-proBNP [3]. The biology of ANP/BNP and NT-proBNP strongly indicate that they are suitable as objective tools for monitoring and managing patients with chronic HF [1].

We have shown in previous publications that a single measurement of plasma NT-proBNP correlates well with the prognosis and survival outcome in CHF [4]. In agreement Januzzi and colleagues consider BNP and NT-proBNP measurements to be the current gold-standard biomarkers for prognosis in chronic CHF [3]. Recent clinical practice guidelines also imply that serial measurements of BNP and NT-proBNP provide valuable information regarding the progression of CHF disease, need for hospitalization and mortality [1].

We have previously studied the peripheral microcirculation as a surrogate for microvascular changes in subjects with different degrees of CHF and of different ages [5, 6]. The cutaneous microvascular responses to iontophoretic administration of the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent vasodilator sodium nitroprusside (SNP) were reduced in CHF [5]. These responses were also attenuated by the

age of the subjects; hence it is important to perform experiments in well-matched clinical groups.

Because of the need of novel strategies it has been suggested that BNP could serve as a “novel” type of pharmacological treatment to reduce overload in CHF patients [3, 7]. Thus, recombinant BNP (Nesiritide) has shown vasodilator properties and is approved for use in acute CHF [8, 9]. However, a recent large randomized study showed in patients with acute decompensated heart failure no change in rate of death and hospitalization, and had no significant effect on dyspnoea [7]. Because of highly elevated levels of endogenous BNP in severe CHF, we hypothesize that administration of exogenous BNP would be unsuitable for treatment of severe heart failure patients probably due to downregulation of natriuretic receptor function. It is known that BNP receptors can downregulate following constant exposure to BNP [10] and this may occur in CHF patients.

The aim of this study was to investigate if responses to BNP in the peripheral microcirculation are altered in CHF patients. We also compared vasodilatory blood flow responses of BNP with that of ACh and local heat.

## **Methods**

### **Patients**

Group 1 consisted of 15 patients with CHF, 9 men and 6 women, mean age of 77.8 years. They were diagnosed earlier with chronic congestive heart failure (CHF). Due to worsening of the condition, they were admitted to the emergency ward clinic at Lund University hospital, Lund University, Sweden, with New York Heart Association (NYHA) class III/IV symptoms and NT pro-BNP levels  $\geq 3000$  ng/L.

Group 2 consisted of 10 healthy elderly age- and gender-matched subjects recruited from the community registry. These subjects had a mean age of 78.8 years of age. Their NT pro-BNP levels were in the normal range; varying between 50 to 450 ng/L. They did not take any medication for cardiovascular disease. For demographic details of the two groups of subjects see Table 1. We found no difference between them in general parameters.

The chronic congestive heart failure patients had reduced left ventricular function as assessed by echocardiography and were all non-current smokers when entering the clinical study to avoid any effects on flow measurements [5]. All patients were kept on their prescribed medication but refrained from long-lasting nitrates 6 hours before the Laser Doppler blood flow measurement. No other co-morbidity resulted in exclusion of participation in the study; only tremor was considered not suitable for the laser Doppler blood flow method. For demographic details on the subjects, see Table 2.

### **Ethics**

The investigation conformed to the principles outlined in the Declaration of Helsinki, Seoul 2008. The Ethics Committee of Lund University approved of the protocol (D.No: 2012/224). Written informed consent was obtained from all patients and healthy controls by the investigators before they were entered into the study and this was verified in the electronic medical charts.

### **Clinical parameters**

Hemodynamic measurements consisted of arterial blood pressure and heart rate. Blood pressure was measured non-invasively in the supine position from the upper left arm with the

cuff inflated at heart level. Blood pressure was taken after the blood flow measurement when the patients had been resting for about 1 hour. The diastolic value was accepted as Korotkoff's phase V. All blood pressure measurements were taken by the same investigator. Heart rate was counted for one minute (see Tables 1 and 2).

### **Blood analysis**

Plasma levels of inflammatory markers, C-reactive protein (CRP), cytokines; interleukin (IL) 6 and soluble IL 2 receptor (s-IL2r) were measured as well as pro-brain natriuretic peptide (NT-proBNP), and blood glucose levels. In addition, plasma levels of haemoglobin (Hb), sodium, potassium, creatinine and cystatin-C, and uric acid were analyzed at the Department of Clinical Chemistry and Pharmacology, Lund University Hospital. Interleukins were measured at the Clinical Immunology laboratory at Lund University Hospital. All blood samples were obtained from peripheral venous access in heart failure patients and in the controls and measured by validated techniques. For details see Table 3.

### **Blood flow measurements**

Cutaneous blood flow was measured using the PeriFlux system 5000 (Perimed, Järfälla, Sweden). This method is non-invasive and gives minimal discomfort to the patients which make it suitable for severely ill patients at bedside [11]. Laser-generated light at a wavelength of 780 nm is directed to the skin using a fibre optic probe. The light reflected from moving blood cells in the superficial skin microvessels undergoes a shift in frequency (Doppler Effect) that is proportional to the number and velocity of moving blood cells. The laser-Doppler output is semi-quantitative, and we have presented all data as the percentage change compared with the baseline perfusion value. Temperature of the skin was recorded continuously.

### **Laser Doppler calculation**

Light is transmitted to the tissue via a fibre-optic probe. When the light hits moving blood cells, it undergoes a change in wavelength (Doppler shift). The magnitude and frequency distribution of these changes are directly related to the number and velocity of blood cells, i.e. the blood perfusion. Measurements are expressed in arbitrary Perfusion Units (PU). Full linear correlation to absolute perfusion value is achieved using Perimed's analysis technology (including a linearization function to avoid underestimation in highly perfused tissues) and calibration using automatic instrument zeroing and Perimed's Motility Standard.

The responses are expressed as the maximum percent change in PU from baseline flow to the iontophoretic administration of ACh and BNP. The perfusion change after local heating (e.g. +44 °C) is a measure of the tissue reserve capacity.

### **Iontophoresis**

Constant current iontophoresis was used to enhance the perfusion of charged molecules into the skin of the dorsal side of the lower arm. The Perilont System (Perimed) used in this study consists of an applicator with a small recess in the centre and a circular temperature probe surrounding the application site. The recess in the centre allows the insertion of a fibre optic probe to measure the blood flow in the stimulated area. An additional temperature probe containing a fibre optic probe was placed at a distance suitable to avoid large veins. This was used as a reference during the iontophoresis and was subsequently used to determine the response to local warming.

Endothelium – dependent vasodilatation was evoked by iontophoresis of acetylcholine (ACh; 2% dissolved in MilliQ water; Sigma, St. Louis, MO, USA.) using anodal current to deliver

the positively charged molecule. Brain Natriuretic Peptide-32 human [BNP; molecular weight: 3464.05 (Batch No.1A, TOCRIS bioscience, UK); 0.05% dissolved in MilliQ water], is also a positively charged molecule and was delivered using the anodal current.

### **Protocol**

All studies were performed at room temperature (+22 – 24 °C). For the severely ill CHF patients, the measurements were obtained at bedside at the hospital internal medicine ward. For the healthy subjects, blood flow measurements were carried out at the emergency medicine ward, MAVA, Lund University Hospital, Lund, Sweden. All subjects were resting in a supine position. Blood pressure and heart rate were measured before and after stimulation and the lowest value is given. The skin of the dorsal lower arm was gently cleansed and the iontophoretic applicators/fibre optic probes were applied to the forearm resting on a pillow to give comfort and provide stabilization. The basal blood flow was studied for 2 min after which ACh was transferred by iontophoresis (anodal current, 0.2 mA for 20 s). The current alone did not affect the blood flow (results not shown). The protocol was based on our previous studies [6] when we determined that successive iontophoretic stimuli at 60 s intervals, produces a cumulative stimuli-response curve. We measured the maximum response after 5 stimuli. The vasodilatory effect was studied by iontophoresis of BNP as above (anodal current, 0.2 mA for 60 s). The stimulation was repeated four times at 60 s intervals. Finally, the response to heat was measured following local warming to +44 °C for 10 minutes. This response was considered as maximum vasodilatation in the microvessels of the skin using this technique.

### *Effect of L-NAME*

L-N-Arginine-methyl-ester (L-NAME; 2%; Sigma, USA) was administered by iontophoresis to 3 healthy persons by a separate protocol to test for effects on the BNP response. First, BNP 0.05% was given with 0.2mA current for 1 minute and repeated for 4 stimulations. After that, L-NAME 2% was given with 0.1mA current, for 1 minute and repeated 4 times on the dorsal side of the lower arm skin area. Then BNP was administered once more and with the same procedure as above and on the same probe site of skin area where L-NAME was given.

### **Statistical analysis**

Statistical analysis was performed by Mann-Whiney *U* test. Statistical differences with a *p* value < 0.05 or less were considered significant. Calculations were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

## **Results**

### *Basic characteristics*

There was no significant difference between the two groups, the healthy aged and the heart failure patients regarding gender, age, BMI, pulse rate and blood pressure (Table 1).

The heart failure patients consisted of 5 subjects with NYHA III and 10 subjects with NYHA IV. The healthy group did not have any cardiovascular diagnosis. The diagnosis of the heart failure subjects were based on symptoms but also in 11/15 patients, a chest X-ray showed significant pulmonary oedema. EKG showed atrial fibrillation in 12/15 of the subjects. The ejection fraction was reduced and showed a mean of 37.7%. Treatment in this group of CHF patients was dominated by ACE inhibitors, beta-adrenoceptor blockers, diuretics, ASA or warfarin. None of the healthy aged controls had these medications (Table 2).

As expected, blood samples showed much higher levels of NT- proBNP in the CHF group ( $5286 \pm 893$  ng/L) as compared to the healthy controls ( $251 \pm 85$  ng/L;  $p < 0.05$ ). The CHF group also showed significantly higher levels of CRP ( $p < 0.001$ ), creatinine ( $p < 0.01$ ), uric acid ( $p < 0.001$ ), IL-6 ( $p < 0.001$ ) and soluble IL2r ( $p < 0.001$ ). These represent indications of a pro-inflammatory state in CHF subjects (Table 3). The calculated absolute glomerular filtration rate [12] was  $42.1 \pm 4.6$  mL/min in NYHA III/IV and  $64.8 \pm 4.2$  mL/min in the aged healthy controls ( $p < 0.05$ ).

#### Microvascular responses

The basal LDF values in PU at rest did not differ between the two groups ( $p > 0.05$ ). The increase in local skin temperature (Heat) caused a maximum dilatation and this was used as a way to cause near maximum dilation of the local cutaneous vascular bed [6]. We found that the response to heat was increased by  $1484 \pm 118\%$  relative to baseline in the healthy controls. In the CHF group, the response to heat was lower ( $959 \pm 114\%$ ;  $p < 0.05$ ) as compared to healthy controls.

ACh stimulates the release of NO and results in subsequent dilatation of cutaneous blood vessels. Following iontophoretic application of ACh, the control subjects showed a mean increase in blood flow of  $1041 \pm 154\%$ ; the CHF patients showed a lower mean increase to ACh administration of  $727 \pm 96\%$  ( $p < 0.05$ ).

#### Response to BNP

The responses to BNP in the controls were more variable than responses to ACh (Figure 1). Flow was increased by  $908 \pm 178\%$  in the healthy controls. There was a markedly lower response to BNP in the CHF patients ( $251 \pm 32\%$ ;  $p < 0.001$ ) as compared to controls. The

increase in PU units to the BNP administration was  $61.4 \pm 22.0$  in healthy individuals and  $20.3 \pm 5.0$  in CHF patients ( $p < 0.05$ ). The increase in response to ACh and heat did not reach significance between the two groups in absolute PU units.

In order to examine if BNP mediates its effects via an endothelial mechanism, the nitric oxide synthase inhibitor L-NAME was tested. The relaxant response to iontophoretically administered BNP was tested in the microcirculation before and after L-NAME treatment. BNP (studied in 3 healthy subjects). We found that BNP elicited an increase of blood flow of  $1280 \pm 127\%$ . L-NAME alone had almost no effect ( $25 \pm 5\%$  change in PU). When BNP was given in the presence of L-NAME, its effect was markedly reduced ( $109 \pm 9\%$  increase in flow;  $p < 0.05$  compared to BNP alone). These data show that the vasorelaxation of BNP is dependent on production of NO.

#### Gender aspects

There were no significant differences in the relaxant responses to ACh, BNP or heat between males and females in either the healthy controls or in the CHF subjects. For example ACh, increased flow by 751% in male patients and 692% in females. For BNP in CHF subjects, the flow response was 356% for men and 303% for women.

## **Discussion**

The present study shows for the first time that patients with severe CHF have reduced microvascular reactivity to BNP. To study this we used a non-invasive method combining iontophoresis drug administration with laser Doppler flow measurements to assess the vasomotor reactivity of cutaneous blood vessels in ill and healthy elderly subjects [11]. The CHF patients showed highly elevated blood levels of NT-proBNP, in agreement with what we and others have found previously, due to enhanced formation and release from stressed myocytes in the hearts of CHF patients. The findings are consistent with the hypothesis that chronic exposure to circulating BNP downregulates the vasodilator response to BNP in the peripheral vasculature of CHF patients. Kuhn [13] observed in myocardial biopsies from CHF patients that ANP and BNP were markedly elevated (30-fold) while the natriuretic peptide receptor type C (NP-C) was only 4-fold increased. This may seem in opposition to our observation but the function of the regulatory element guanylyl cyclase-A (GC-A) was abolished in severe CHF [13]. The findings suggest that therapeutic use of BNP or natriuretic peptide analogues may be limited due to decreased effectiveness in patients with advanced heart failure.

Early studies on BNP showed that it was a dilator of different vessel types in the circulation [3]. Our study is the first to show that BNP also acts as a vasodilator in the cutaneous microcirculation of humans in aged and severe CHF patients. Under normal conditions or in early stages of CHF, peripheral vasodilatation by BNP (and ANP) is likely beneficial to reduce overload when the heart experiences stress. However, our study indicates that the effectiveness of this mechanism may decline with advanced heart failure. In situation of left ventricular assisted device (LVAD) use some reversal may occur which could indicate a way to counterbalance the refractoriness of systemic BNP [13].

The reduction in BNP-mediated vasodilatation may be due to several interacting mechanisms: (i) BNP may act on all three subtypes of natriuretic peptide receptors (NPR-A, -B and -C) so alterations in or more of these may exist. The present study did not quantify the natriuretic peptide receptor protein expression which is a future project. (ii) The hypothesis that the vascular natriuretic receptors have been desensitized by chronic exposure to high levels of circulating natriuretic peptides in CHF patients is supported by the literature [13, 14]. This may occur at different sites such as at the receptor or on the function of the receptor. BNP acts on specific natriuretic receptors: NPR-A and NPR-B, guanylate cyclase-linked receptors, and NPR-C, a G<sub>i</sub>-protein-linked receptor [9]. The latter receptor is likely responsible for the nitric-oxide mediated dilation observed in the present study [1]. (iii) Although it has been found that CHF induces increase in NPR-C mRNA in heart biopsies [13, 14] this is not equivalent to demonstration of actual receptor reduction because this must be shown by protein or functional quantification. Clearly this fact needs future demonstration.

An alternative explanation for the reduced BNP response is that, in elderly subjects, there is a general decline in vascular responsiveness with aging. In a previous study, we showed that the microvascular relaxant capacity in skin vessels is reduced in healthy elderly people and attenuated further by the presence of heart failure [6]. Nevertheless, the BNP response measured here in the healthy elderly controls was reasonably robust. Moreover, the cutaneous microvasculature of controls, as well as in the CHF patients, dilated appropriately to a local heat stimulus. While the heat response was somewhat diminished in the CHF patients relative to controls, it was less affected than the BNP response. This suggests that other mechanisms underlie the larger reduction in BNP-mediated vasodilatation (72%) as compared to about 30% in heat or ACh responses.

Another possible reason for the diminished BNP response in CHF patients could be that specific dilator signalling mechanisms are blunted in heart failure. Using the nitric oxide synthase inhibitor, L-NAME, we showed here that dilation to BNP was dependent on nitric oxide formation in human cutaneous microvessels. L-NAME iontophoresis has recently been validated as a tool to assess NO-mediated dilatation in humans [15]. We have recently performed a study of isolated human subcutaneous arteries where we also find that BNP acts via an endothelium-dependent dilator mechanism (Edvinsson, unpublished data). To further assess the nitric oxide pathway for vasodilatation, we measured blood flow in response to ACh, a well-established dilator that activates endothelial nitric oxide synthase in a manner similar to BNP. There was some reduction in ACh dilation in CHF patients; however, this response was not affected to the same degree as that of BNP in the present study. In a comparative trial of nesiritide and nitroglycerine on the effectiveness of vasodilatation in the management of acute CHF the outcome showed weaker effect of BNP than of nitroglycerine [9].

CHF subjects are typically of advanced age; in agreement the mean age of the present CHF patients was 77.8 years and controls 78.8 years, hence they were well matched. This is important because we have previously found that cutaneous vascular reactivity is reduced by age and is even further attenuated by the presence of heart failure [6]. Gender differences were another consideration; little is known regarding possible sex differences in the peripheral microvasculature of CHF patients. In the present study there were 9 male and 6 female patients, which is reflective of the gender distribution observed generally in CHF [16, 17]. In the Swedish Heart Failure registry, a large CHF cohort of 50,827 patients, the age group 75 - 84 years contains 59 % males and 41% females [16, 17]. We found no sex differences in

cutaneous microvascular Laser Doppler flow in response to BNP, ACh or heat in CHF and control subjects of similar age; hence it is justified to group them together.

The reason for the reduction in responses of the subcutaneous microcirculation could in part be associated with a low degree of inflammation. In our CHF patients the inflammatory cytokines IL6 and IL2 receptor, C-reactive protein and uric acid were all highly elevated compared to controls. Increases in these circulating markers of inflammation have been reported in earlier studies of CHF [6, 18]. The IL2 receptor is of particular interest as blood levels of this marker seem to be linked specifically to CHF and may be useful as a diagnostic measure to follow the progression of CHF. At this point, we do not know how inflammation may influence the vasodilatory capacity of the cutaneous microcirculation in CHF, but this point needs further investigation.

### **Limitations of the study**

Although the number of patients in this study was not large, the values were clearly significant. Hence more patients would not change the general concept or conclusions. Medication is another consideration in evaluating responses of CHF patients. The drug profiles for the CHF patients in this study were in accord with the standard recommendations for this disease. However, because of the severity of their condition, they were not always on the optimum doses recommended for CHF treatment. Particularly in elderly subjects, they cannot tolerate the high levels of beta-adrenoceptor blockade and vasodilators due to reductions in many compensatory systems with age. Many of these drugs may affect the vascular system and thus influence measurements of microcirculatory flow. However, it is unlikely these influences underlie the differences between reactivity between BNP and ACh/heat.

In evaluating patient responses, it is also important to keep in mind that CHF patients often have co-morbid conditions that could influence the results. Atrial fibrillation is a common co-morbidity in CHF [17]. In the CHF group, 12 of the 15 patients had atrial fibrillation, and 4 of these had a pacemaker. Clinical parameters of importance such as the QRS complexes with a width > 120 ms have now been shown to be a marker for severity of heart failure with poor outcome and higher mortality rate in CHF. [17].

## **Conclusions**

We have for the first time demonstrated that the cardiac hormone BNP acts on human cutaneous microvessels to increase blood flow via a nitric oxide dependent mechanism. A major finding was that BNP has poor relaxant effects in patients with severe CHF. The mechanism behind this is not known, but may involve receptor downregulation or reduction in guanylyl cyclase activity with reduced formation of cGMP in response to the elevated circulating levels of BNP in advanced stages of CHF.

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## **Conflict of interest**

None

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**Table 1.** The demographics of congestive heart failure patients vs. healthy subjects.

|                          | Heart Failure<br>n=15 | Healthy<br>n=10    |
|--------------------------|-----------------------|--------------------|
| Sex (F/M)                | 6/9                   | 5/5                |
| Age                      | 77.8 ± 1.5 (77–89)    | 78.8 ± 1.2 (72–85) |
| BMI (kg/m <sup>2</sup> ) | 26.5 ± 1.47 (18–40)   | 23.2 ± 1.0 (20–29) |
| BP Syst (mm Hg)          | 125.0 ± 4.8           | 131.8 ± 5.1        |
| BP Diast (mm Hg)         | 72.1 ± 4.0            | 73.3 ± 2.6         |
| Pulse/min                | 79 (67–92)            | 67 (55–72)         |

Data given as mean ± S.E.M., and/or range in parenthesis. No statistical differences with Mann-Whitney's nonparametric test were found between Heart Failure and Healthy subjects. BMI: Body mass index; BP: blood pressure; Syst: systolic; Diast: diastolic.

**Table 2.** Medical history and treatment of chronic congestive heart failure patients

|                                 | Heart Failure<br><i>n</i> = 15 | Healthy<br><i>n</i> = 10 |
|---------------------------------|--------------------------------|--------------------------|
| NYHA III                        | 5                              | n/a                      |
| NYHA IV                         | 10                             | n/a                      |
| Pharmacological treatment       |                                |                          |
| Beta-adrenoreceptor antagonists | 13/15                          | n/a                      |
| ACE-inhibitors                  | 11/15                          | n/a                      |
| ARB                             | 3/15                           | n/a                      |
| Diuretics                       | 14/15                          | n/a                      |
| Diogoxin                        | 1/15                           | n/a                      |
| Spironolactone                  | 0/15                           | n/a                      |
| ASA                             | 8/15                           | n/a                      |
| Warfarin                        | 9/15                           | n/a                      |
| Chest X-ray                     |                                |                          |
| Pulmonary oedema                | 11/15                          | n/a                      |
| Electrocardiogram               |                                |                          |
| Atrial fibrillation             | 12/15                          | n/a                      |
| Pacemaker                       | 4/15                           | n/a                      |
| QRS complex width (ms)          | 115.5 ± 8.6                    | n/a                      |
| Ejection Fraction (%)           | 37.7 ± 1.9                     | n/a                      |

*n*: number of patients; NYHA: New York Heart Association classification; ACE: angiotensin converting enzyme; ASA: acetylsalicylic acid; ARB: angiotensin receptor blockers; QRS widths (ms); EF: ejection fraction (%).

**Table 3.** Laboratory blood analysis (mean  $\pm$  S.E.M.).

|                            | Heart Failure<br><i>n</i> =15 | Healthy<br><i>n</i> =10 |
|----------------------------|-------------------------------|-------------------------|
| NT-proBNP (ng/L)           | 5286 $\pm$ 893*               | 251 $\pm$ 85            |
| Hemoglobin (g/L)           | 121 $\pm$ 3.6                 | 130 $\pm$ 1.5           |
| Sodium (mmol/L)            | 141 $\pm$ 1.1                 | 141 $\pm$ 0.6           |
| Potassium (mmol/L)         | 4.0 $\pm$ 0.1                 | 4.2 $\pm$ 0.1           |
| Creatinine ( $\mu$ mol/L)  | 132.2 $\pm$ 12.1**            | 78.8 $\pm$ 4.1          |
| Uric acid ( $\mu$ mol/L)   | 579 $\pm$ 40***               | 281 $\pm$ 21            |
| CRP (mg/L)                 | 15.0 $\pm$ 3.7***             | 1.4 $\pm$ 0.3           |
| IL-6 (ng/L)                | 28.2 $\pm$ 10.6***            | 3.6 $\pm$ 0.5           |
| IL-2r (kU/L)               | 967 $\pm$ 131***              | 406 $\pm$ 39            |
| eGFR <sup>a</sup> (ml/min) | 42.1 $\pm$ 4.6*               | 64.8 $\pm$ 4.2          |

NT-proBNP: Nerve terminal-pro-brain natriuretic peptide; CRP: Sensitive C Reactive Protein; IL: Interleukin; IL-2r: Soluble IL 2 receptor; eGFR: estimated glomeruli filtration rate (Cockcroft-Gaults adults). Statistical analysis was performed using the non-parametric Mann-Whitney's test. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared to healthy subjects.

## Legend to illustration

**Figure 1.** Microvascular relaxant responses in healthy individuals (n=10) and of patients with congestive heart failure of NYHA class III-IV (n=15). Shown are the endothelium-dependent responses to acetylcholine (top), the relaxation response to BNP (middle) and the general vasodilator response to local heating to +44 °C (bottom). Values represent mean maximum increase in blood flow  $\pm$  S.E.M., n = 10 – 15 subjects, \* =  $p < 0.05$ , \*\*\* =  $p < 0.001$  relative to healthy age-matched controls.

