Natriuretic Peptides and the Microcirkulation in Heart Failure Patients

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2014

Natriuretic Peptides and the Microcirculation in Heart Failure Patients

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LUND
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DOCTORAL THESIS
By due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended at Segerfalksalen, Wallenberg Neuroscience Center, Lund, Sweden.
Date 21st of February 2014, at 13.00 pm.

Faculty Opponent

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Organization LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences, Lund Department of Emergency Medicine	Document name: DOCTORAL DISSERTATION
	Date of issue: 21 february,2014
Author(s) Marie-Louise Edvinsson	Sponsoring organization

Title and subtitle: Natriuretic peptides and the microcirculation in heart failure patients

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.

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.

Paper I: We investigated the prognostic value of NT-proBNP for monitoring progression of HF. High blood levels (>5000) were found to indicate a poor prognosis of HF.

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.

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.

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.

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.

Key words: Heart failure natriuretic peptides BNP cutaneous microcirculation

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language: English
ISSN and key title:1652-8220	ISBN:978-91-87651-29-8
Recipient's notes	Number of pages 122 / Security classification	Price

Natriuretic Peptides and the Microcirculation in Heart Failure Patients

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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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ISBN 978-91-87651-29-8
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2013

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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, hjärtmuskel inflammation 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 ansträngersig, 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 kärldysfunktion 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 kärldysfunktion.

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 hjärtsviktspatienter 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 hjärtsviktspatienter 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
TNFα	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
Class I (Mild)	No limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnea (shortness of breath).
Class II (Mild)	Slight limitation of physical activity. Comfortable at rest. But ordinary physical activity results in fatigue, palpitation, or dyspnea.
Class III (Moderate)	Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity causes fatigue, palpitation, or dyspnea.
Class IV (Severe)	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

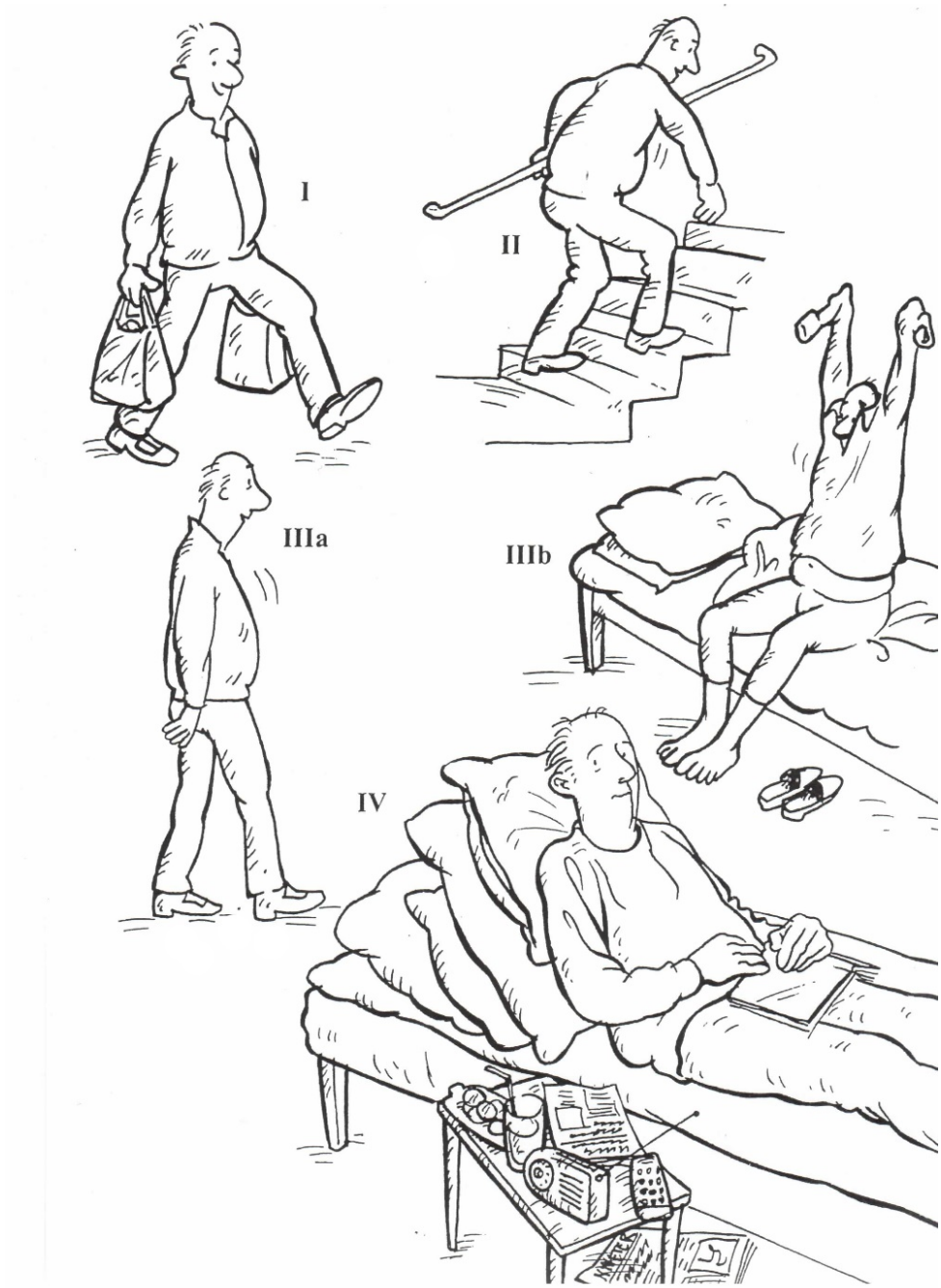

Figure 1
NYHA Classification – The Stages of Heart Failure. This syststem relates symptoms to everyday activities and the patient's quality of life. Image with permition from Hässle Läkemedel AB.

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).

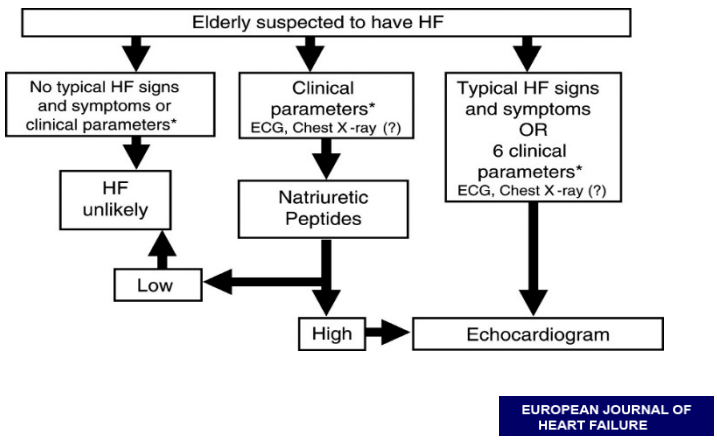

Figure 2
Potential algorithm for the diagnosis of heart failure in the elderly with increase in age, loss of appetite, absence of wheezing, low body mass index and nocturnal dyspnoea.(Parissis et al. 2002).

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 effected 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 artrial 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 hyporesponsivness 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 $B_{6,}B_{12}$, 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 $B_{6,}$ B_{12} 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 18th 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 20th 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) wich 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^2 (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^2. 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^-) and positive (Na+) 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^2.Tthis 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.

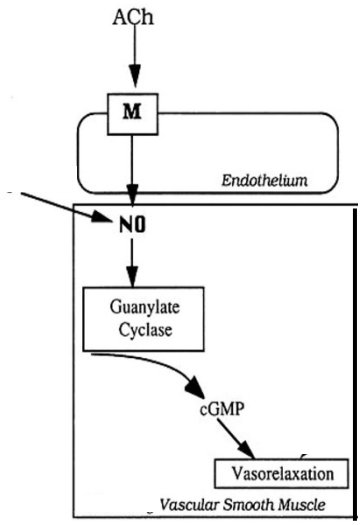

Figure 4
Sodium nitroprusside acts like an NO doner, which then acts via cGMP to dilate the vasular smooth muscle.

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 where 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° 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.

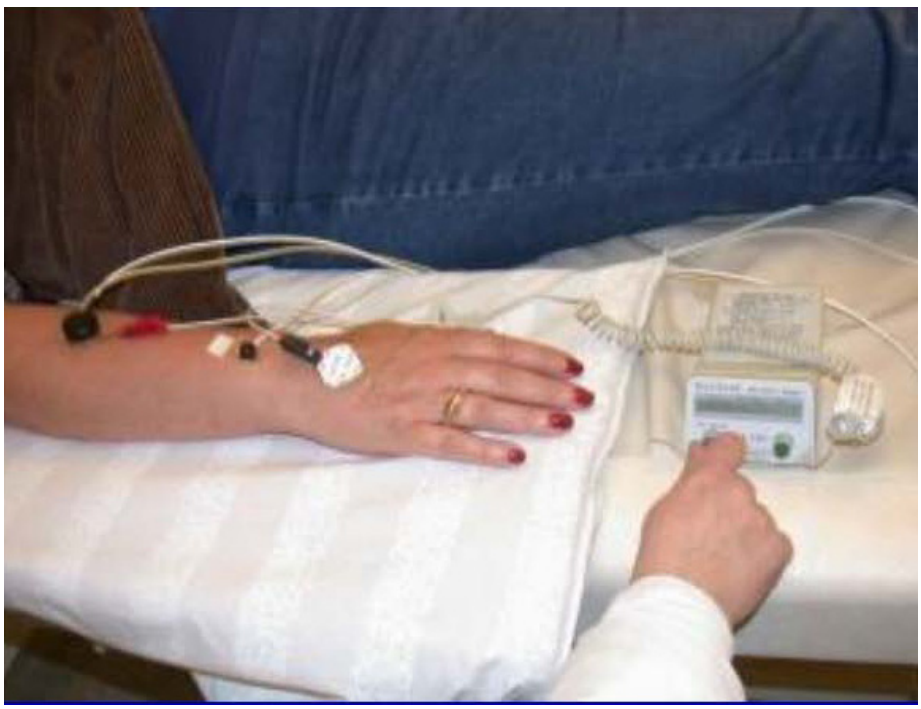

Figure 5
The Periflux 5000 Sytem with the big black probe containing the substance of choice, here acetylcholine, beacuse it is connected to the red crocodile wire (anode). The smaller probe (in the center) with a laser light is now acting as a controle mesurement of the blood flow. To the right we see the instrument of the battery were 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).

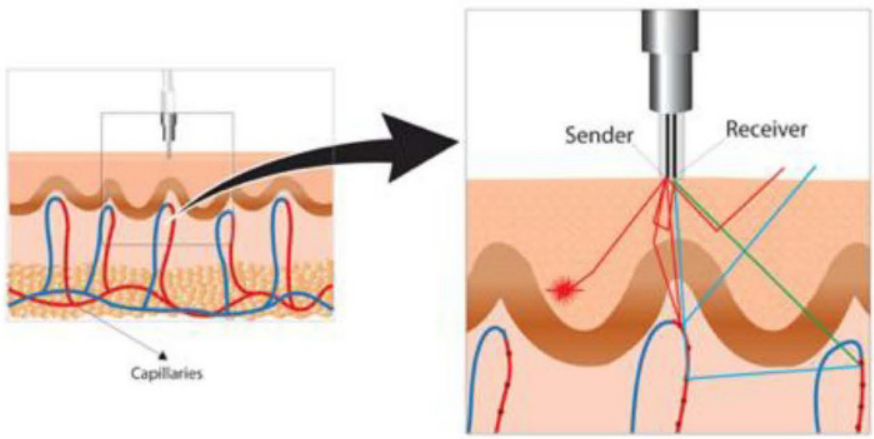

Figure 6
The principle of the Laser Doppler technique for assesing blood flow is to measure the Doppler shift – the frequency change that light undergoes when reflected by moving objects, such as red blood cells. Measurement of the RBC motion is recorded continuously in the outer layer of the tissue. The number of red cells times their velocity is reported as microciculatory 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 μM, 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_6, B_{12} 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 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 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_{12} 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 ± 4 years), heart failure patients with New York Heart Association class IV (NYHA IV) and with a NT-proBNP level ≥ 5000 ng/L; Group 2 (76.5 ± 2 years), heart failure patients with NYHA II and NT-proBNP ≤ 2000 ng/L, and Group 3 (67.6 ± 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-α, 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 foresay 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.

Acknowledgements

I wish to express my gratitude to all my patients who willingly gave their consent and to my healthy control persons who contributed to this PhD thesis. I would like to thank the following people in particular:

First of all I would like to thank my supervisor ***Sven E. Andersson,*** for agreeing to take me on as an apprentice and for being my tutor and friend. We have now done research together for more than a decade and I cannot think of any time that we have been in disagreement. You only very mildly suggest that I read this and that, article and you always deliver answers to my many questions. I have a great admiration for you being so updated in the vast field of HF. Our teamwork is close to perfect, being only the two of us – there is no room for not doing your share of the work. Thanks for many nice conversations over a beer talking about dogs.

My thanks to my co-supervisor, ***Erik Uddman,*** whom I have known for a long time, but who came in my research period later. Thanks for taking me through my computer hesitation and helping me by the hand. Thank you for your participation in design and carrying out the clinical studies.

Diana Krause, my dearest over-see´s friend, my sister in science. No one can like you, correct my English or re-write a whole chapter without me shredding a single tear. Thank you for all your help with manuscripts, presentations and for endless advice. You are always teaching me to be prepared. Thanks for our deep talks, all thru the nights, until dawn, in our summer house, for your generous hospitality, and help at numerous occasions, when we stayed with you in California. Thanks for all the love and support, which makes me dare to go even further. "California dreaming"….

Elisabeth Nilsson, for your excellent skills of preparing acetylcholine and other substances and for always deliver them promptly, for being the natural organizer of Lars' lab. Thank you for many nice chats and laughter's.

Hilda Ahnstedt; Thanks for being the inspiration of positive thinking at the lab, for your generosity, and always without a hesitation, helping all of us. In particular and especially for me, thanks for help with figures, tables, computer, coffee machine, buying milk, technical and practical help. Thank you Hilda, I could not have done this without you. "Don't go changing"……

I would like to acknowledge my co-authors at this point, since you have contributed to published papers even outside this thesis. ***Susanne Bondesson, Thomas Pettersson,***

Christine Bengtsson, Ola Nived, Gunnar Sturfeldt, Ulf Ekelundh, Ivan Dimitrejevic, P-O Kimblad, Malin Malmsjö, Isak Lindstedt, Jonas Björk and Lars Edvinsson.

Karin Warfvinge, for giving me "the heart", immunohistochemistry picture that covers my thesis. A technique that I understand that you master to perfection,"world class". It must have been fate that wanted our roads to cross, and I am so glad it did. Thank you for all our deep philosophical conversations, analysis and good laughs.

I want to thank all former researchers at Lars lab, Experimental Vascular Research, for many nice PhD parties over the years, now more than 50! To the present "crew" I would like to express my deepest thanks for helping me a lot in my writing-up period with technical support in writing and with the coffee machine and for small talk at breaks. For providing the right atmosphere so that I can get in the mood of writing the "Kappa". When I think of all the champagne at "Nailings" I realize that is quite a lot, but it is a very nice habit.

There are so many papers and dates to keep in mind when you are a PhD student and without the excellent help from ***Maj-Lis Olsson*** and ***Helene Johbonell,*** I would not have figured it out. Many thanks for your administrative skills and the entire "extras" you have done for me, helping out with receptions, parties and education.

My sincere thanks; to ***Solveig Wennerholm,*** for teaching me all about CRF, case report forms, SOP, standard operating procedures, and GCP, good clinical practice, when I started with clinical trials and who has been an inspiration for managing clinical research in hospital and how to "monitor", studies with high quality standard.

To Professor ***Ulf Dahlström***, chairman and "keeper" of RIKS SVIKT, Swedish register of Heart Failure patients, and ***Åsa Johnsson,*** coordinator, for sharing their vast knowledge and engagement in the field. Thanks for inviting me onto the "board of steering committee". Thank you for fruitful conversation and the "latest news" in the field of HF.

My gratitude to "Akutklinken", Lund University Hospital, SUS, that by financial and practical support made the progress of this thesis possible.

To my colleagues at the Emergency, MAVA, KAVA and Internal Medicine.

To my colleagues in Education and clinical teaching.

To colleagues and friends at "kaffebordet", former stab of the Emergency clinic, for all nice conversations of different subjects and for our most important, Friday breakfasts – the highlight of the week.

To ***Kristina Engels, Eva Knutsson, Åse Jönsson and Margareta Kjäll,*** my "life-line", dear friends and colleagues from our year at LIV – leadership in healthcare course. Thank you for always being there for me in stressful times and for knowing what I mean, when bringing up old phrases and symbols like "inductive synthesis, and Christmas evangelism drama. Flying an airplane, filming, drama, mission impossible

IKEA administration, Friday-conferences politicians, Bishops, interviews, ice wakes, Human Relations. You are my true network.

To my truly best friend, ***Helene Rafstedt Johnsson,*** specialist in pediatric nursing and my "brother in arms". We know each other inside out. It is fantastic to have that friendship, and together with you I can always just be myself. You are always promoting whatever I do, always willing to help me out, always "on your toes". How can I ever fail with a friend like you? Forever, yours.

To my sisters, ***Kristina Linderoth and Eva Hagenborn,*** and their families, you are so dear to me and we always get along fine, without jealousy. You are no further away than a phone call.

To my mum and dad, ***Ethel and Bror Linderoth,*** thank you for bringing me up to be curious and letting me try new things. And for letting me know that I could always come home if I did not succeed. You taught me to do my best at work.

To my family: our sons,

Johan Edvinsson, -an Officer and a gentleman. You are always willing to help others, polite and thoughtful, enthusiastic, cheerful and trustworthy. Whenever I am troubled you can analyze the problem and provide a plan and then I am calm again. I knew you were a leader from the moment I laid eyes on you. You and I share the same vision of team work-that truth that the chain is not stronger than its weakest spot. I am so proud of you and I love you very much. Our song "you're the meaning of my life, you're the inspiration"…

Jacob Edvinsson, - the philosopher and the analyst of the family, now doing very well in chemistry and pharmacy studies. Thanks for your wittiness and humoristic talents. You take such good care of your friends, customers and patients. Thank you for having patients with me when showing me how to handle computers and all other technical things. You are so caring and your fondness for animals and nature has been there from the start. Thorough, responsible and well prepared are also words that come to mind, describing you. This last year, you have really grown up and I am so happy for your new "era" in Göteborg, being and entertainer and singing tenor. I realize that you are not my little boy anymore but I am very proud of your change and love you very much. Our song-"I will take you in little sparrow"….

To ***Lars Edvinsson,*** my beloved husband, to whom I owe everything in my scientific development. You are literarily always there for me, with endless patients, and always "rowing the boat to shore". You are my safety in life. Words are limited here. So I will just say thank you. I love you very much. Our song "Love for ever-true"…

I did it my way.

Frank Sinatra

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