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# Left Ventricular Function in Elderly Men

*Metabolic, Hormonal, Genetic and Prognostic Implications*

BY

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## Abstract

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Heart failure and left ventricular dysfunction are major causes of morbidity and mortality. In this thesis, metabolic, hormonal, genetic and prognostic aspects of echocardiographically determined left ventricular function were investigated in a fairly large longitudinal population-based study of men. The participants were examined both at age 50 and 70 years and were followed for mortality using the national cause-of-death registry.

Several factors associated with the insulin resistance syndrome predicted left ventricular systolic dysfunction independent of myocardial infarction, hypertension, diabetes and the use of cardiovascular medication after twenty years follow-up. Plasma levels of N-terminal atrial natriuretic peptide (N-ANP) were significantly increased in men with left ventricular dysfunction in comparison to healthy men. However, the diagnostic accuracy was poor due to the extensive overlapping between the groups. Relations between a haplotype of the novel hUNC-93B1 gene and the E/A-ratio were found and validated in separate samples of the cohort. Myocardial performance index (a Doppler derived index of combined left ventricular systolic and diastolic function) and left ventricular ejection fraction were found to be predictors for cardiovascular mortality independent of traditional cardiovascular risk factors in a longitudinal analysis with a mean follow-up of seven years.

In conclusion, this thesis showed that left ventricular function is influenced by metabolic, hormonal and genetic factors and that echocardiographic measurements of left ventricular function, such as the myocardial performance index, are strong independent risk factors for cardiovascular mortality in elderly men.

*Key Words:* echocardiography, heart failure, left ventricular function, genetics, mortality, insulin sensitivity, natriuretic peptides

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‘What becomes of the brokenhearted?’

*Jimmy Ruffin, Motown Records 1966*

# Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Ärnlöv J, Lind L, Zethelius B, Andrén B, Vessby B, Lithell H, Several factors associated with the insulin resistance syndrome are predictors of left ventricular systolic dysfunction in a male population after twenty years of follow-up. American Heart Journal 2001; 142: 720-24. *

II. Ärnlöv J, Lind L, Stridsberg M, Andrén B, Lithell H, N-terminal atrial natriuretic peptide and left ventricular geometry and function in a population sample of elderly males, Journal of Internal Medicine 2000; 247: 699-708†

III. Ärnlöv J, Sundström J, Lind L, Andrén B, Andersson M, Reneland R, Berglund L, Kashuba V, Protopopov A, Zabarovsky E, Lithell H. hUNC-93B1, a novel gene mainly expressed in the heart, is related to left ventricular diastolic function in elderly men. Submitted for publication.

IV. Ärnlöv J, Lind L, Andrén B, Risérus U, Berglund L, Lithell H. A Doppler-derived index of combined left ventricular systolic and diastolic function is a powerful predictor of cardiovascular mortality in elderly men. Submitted for publication.

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# Abbreviations

| | |
|---|---|
| A | late velocity over the mitral valve |
| 2D | two-dimensional |
| ACE | angiotensin-converting enzyme |
| ANOVA | analysis of variance |
| ANP | atrial natriuretic peptide |
| BMI | body mass index |
| BNP | brain natriuretic peptide |
| CI | confidence interval |
| DNA | deoxyribonucleic acid |
| E | early velocity over the mitral valve |
| EF | ejection fraction |
| ET | ejection time |
| FVI | flow velocity integral |
| ICD | International Classification of Disease |
| ICT | isovolumic contraction time |
| IVRT | isovolumic relaxation time |
| HDL | high-density lipoprotein |
| HUNC | human uncoordinated |
| IVS | intraventricular septal thickness |
| LDL | low-density lipoprotein |
| LVEDD | left ventricular end-diastolic diameter |
| LVESD | left ventricular end-systolic diameter |
| LVOT | left ventricular outflow tract |
| MPI | myocardial performance index |
| N-ANP | N-terminal atrial natriuretic peptide |
| p | probability |
| PW | posterior wall thickness |
| PYAR | person-years at risk |
| ROC | receiver operating characteristic |
| SD | standard deviation |
| SNP | single nucleotide polymorphism |
| TNF | tumor necrosis factor |
| ULSAM | Uppsala longitudinal study of adult men |

# Introduction

Heart failure is a clinical syndrome that can be defined as an imbalance between the ability of the heart to supply blood and the demand placed on it. This condition is among the most frequently encountered cardiac diagnoses and the prevalence and incidence is expected to increase even more[1,2], partly as a result of an ageing population and improved survival after a myocardial infarction.

Heart failure causes substantial morbidity[3], which accounts for a health-care expenditure that is more than twice that of the cost for cancer[4]. Although the therapeutic management of heart failure has improved[5,6], mortality data are comparable to severe forms of malignant disease[7]. In spite of this, our knowledge on the epidemiology of heart failure is scarce, in part due to the fact that heart failure is a heterogeneous syndrome.

There is a discrepancy between the signs and symptoms of heart failure and verification of the heart failure diagnosis using echocardiography. With the increasing availability of echocardiography it has become evident that impaired left ventricular function often occurs without symptoms[8,9] and it is now well recognized that symptomless left ventricular dysfunction exists and progresses a long time before the onset of overt clinical heart failure[10].

The present thesis focuses on left ventricular function, as measured by echocardiography, rather then symptomatic heart failure. A variety of metabolic, hormonal, genetic and prognostic aspects of echocardiographically determined left ventricular function throughout the cardiovascular continuum are addressed in a population of Swedish men.

## Echocardiography and left ventricular function

Echocardiography is the most common method to assess left ventricular function and heart failure. By using echocardiography and Doppler it is possible to examine different features of cardiac function.

*Left ventricular systolic function*

Several echocardiographic indices are used to describe and quantify left ventricular contraction (i.e. left ventricular systolic function). Left ventricular ejection fraction is the most frequently used index of cardiac function and is defined as the stroke volume (the difference between the left ventricular end diastolic and end systolic volumes) expressed as a percentage of left ventricular end diastolic volume. All of the clinical trials in which angiotensin-converting enzyme (ACE) inhibitor and beta-blocker treatment have been shown prolong the life of heart failure patients have used a low ejection fraction as the definition of left ventricular dysfunction[11,12]. Other indices of left ventricular systolic function include cardiac index, atrioventricular plane displacement and left ventricular wall motion score index.

*Left ventricular diastolic function*

Until recently heart failure was almost exclusively regarded as left ventricular contractile dysfunction but there is accumulating evidence that heart failure can also be primarily due to impairment of ventricular filling (diastolic dysfunction). For instance, up to half of the patients diagnosed with heart failure showed an intact left ventricular systolic function in recent population studies[13-15]. Some of the established indices of diastolic function are the ratio between the early and late blood flow velocities over the mitral valve (E/A-ratio) and the isovolumic relaxation time (IVRT), defined as the time between the end of systolic ventricular outflow to the onset of mitral flow.

In clinical practice there is a call for a clinically relevant measurement of cardiac function that is reproducible and easily assessable. As the conventional measurements of left ventricular function, have been shown to have limitations in these respects, new echocardiographic and Doppler indices of cardiac function have been proposed during the last decade.

*Myocardial Performance Index*

The myocardial performance index (also denoted the TEI-Doppler index) is a fairly new measurement of myocardial function and is defined as the sum of isovolumic contraction and relaxation time divided by ejection time[16]. This easily obtainable Doppler index has been suggested to reflect both left ventricular systolic and diastolic function[17] and has previously been shown to have prognostic value in patients with dilated cardiomyopathy, amyloidosis and coronary heart disease[18-21].

*Left ventricular function as a risk factor for cardiovascular mortality*

At present, there are several studies that have identified echocardiographic and Doppler measurements as predictors of cardiovascular morbidity and mortality[21-23]. However, most of these studies have been performed using subjects with symptomatic heart failure or following myocardial infarction, and there are only few studies performed in the general population[24,25].

## Insulin resistance and left ventricular function

Insulin resistance can be defined as a reduced sensitivity in the tissues of the body to the action of insulin. Major cardiovascular risk factors, such as hypertension, glucose intolerance, hyperinsulinaemia, dyslipidaemia and obesity, often cluster in the same individuals, and the existence of an insulin resistance syndrome involving these disorders has been proposed[26].

Heart failure is an insulin resistant state[27-30] and the level of insulin resistance is related to the severity of the disease independently of plasma catecholamine levels and left ventricular ejection fraction[30] and an impaired insulin-mediated glucose uptake has been shown to be an independent prognostic factor in heart failure patients[31].

The components of the insulin resistance syndrome are closely related to the development of coronary heart disease, but little is known about their longitudinal relation to heart failure and left ventricular systolic dysfunction.

## N-ANP and left ventricular function

Atrial natriuretic peptide (ANP) is a cardiac hormone with diuretic, natriuretic and vasodilator activities[32]. ANP is stored as a 126-aminoacid peptide (proANP) in secretory granules of atrial myocytes[33]. ProANP is released in response to atrial stretch[34] and neurohumoral stimulation[35] and cleaved into the biologically active C-terminal end of proANP, ANP (99-126), and the N-terminal end, ANP (1-98) or N-ANP[36]. N-ANP has a reduced clearance rate compared to ANP, which makes the circulating N-ANP concentrations higher than ANP[37].

The circulating levels of ANP have been shown to be elevated in subjects with congestive heart failure[38-44]. Circulating ANP levels have furthermore been shown to be inversely related to both the degree of left ventricular systolic function[41-44] and to survival[45,46] in patients with congestive heart failure. Because of the longer half-life and the greater stability, the plasma N-ANP levels have been suggested to be a more sensitive marker of left ventricular dysfunction than ANP[38,39,44].

Even though echocardiography is the method of choice to diagnose left ventricular dysfunction and hypertrophy, the method is fairly expensive, demands experienced examiners and is not applicable in all patient categories (a proportion of patients with obesity or pulmonary diseases). Therefore, an easily measured circulating substance that is elevated in subjects with left ventricular dysfunction or left ventricular hypertrophy would be an important complement to echocardiography.

## Genes and left ventricular function

There is accumulating evidence for associations between single nucleotide polymorphisms (SNPs) and cardiac function and morphology. To date, mainly SNPs in the renin-angiotensin-aldosterone system and adrenergic receptors have been evaluated in this respect[47-49]. However, there are several examples of conflicting findings, in part due to the combination of various genes and different environmental factors that seems to be involved[50].

*The hUNC-93B1 gene*

The human member of the UNC (uncoordinated) gene family (hUNC-93B1) was recently identified and cloned[51]. hUNC-93B1 is a homologue to the Caenorhabditis Elegans UNC-93 gene, which have been suggested to be involved in excitation-contraction coupling in muscle or in co-coordinating muscle contraction between muscle cells by affecting the functioning of gap junctions[52]. The hUNC-93B1 gene has the highest level of expression in the heart but is expressed in all human tissues[51].

Due to the high gene expression in the heart, we hypothesized that the impairment of skeletal muscle function seen in C. Elegans might also apply to human cardiac muscle and thus affect the function of the heart.

# Aims of the study

In this thesis, metabolic, hormonal, genetic and prognostic aspects of echocardiographically determined left ventricular function were investigated in a longitudinal population-based study of men from Uppsala, Sweden, examined at age 50 and at age 70 years.

The specific aims of the thesis were:

To examine longitudinal and cross-sectional relationships between hemodynamic and metabolic variables and left ventricular systolic dysfunction (Paper I).

To evaluate the association between N-ANP and left ventricular dysfunction and geometry (Paper II).

To explore and validate relations between SNPs and haplotypes of the hUNC-93B1-gene and left ventricular function and geometry (Paper III).

To examine the predictive value of different echocardiographic and Doppler indices of cardiac function for the development of cardiovascular and total mortality (Paper IV).

# Methods

## Subjects

*The ULSAM-study*

All papers in this thesis are based on the Uppsala Longitudinal Study of Adult Men (ULSAM). The ULSAM-study started in 1970 when all men born 1920-24 and living in Uppsala, Sweden were invited to a health survey. Of the 2841 50-year-old men invited, 2322 participated (figure 1). A reinvestigation was performed twenty years later (1990-94) and since the first investigation, 422 men had died and 219 men had moved out of the Uppsala region. Of the 1681 70-year-old men invited to the reinvestigation, 1221 men participated. The 579 first consecutive subjects had an echocardiographic examination approximately within one month of the examinations at age 70 (1991-94). This sub-sample is used in all papers of the thesis. Four-hundred-and-ninety-three of the remaining men had an echocardiographic examination 3-8 years later (1997-2000, median age 75). This sub-sample was used in the validation of the primary results in Paper III.

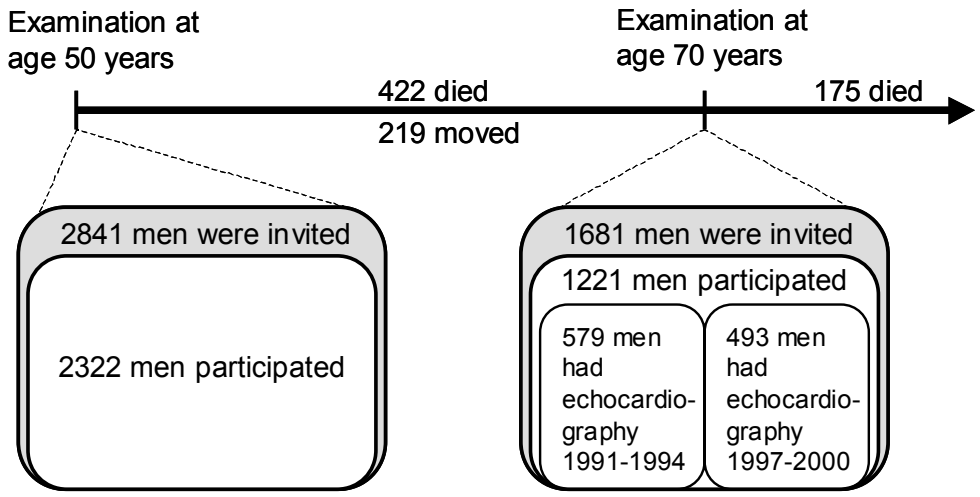


**Figure** 1 Overview of the ULSAM-cohort

All subjects gave their informed consent and the Ethics Committee of Uppsala University approved the study.

*Study populations*

The study populations in paper I-IV were defined at the investigation at age 70 years. See figure 2 for an overview of the different study populations.

**Paper I** The study population in Paper I consisted of the 431 men who had an adequate ejection fraction determination at the echocardiographic examination at age 70 years (1991-94). Left ventricular systolic dysfunction (defined as an ejection fraction ≤ 0.40) was found in 16 subjects. Two control groups were used in a nested case-control design. The first consisting of 48 subjects with normal left ventricular function, frequency-matched on prevalence of myocardial infarction, hypertension and diabetes, as well as on the use of the different cardiovascular medications (ACE inhibitors, calcium antagonists, beta-blockers, diuretics and digitalis). The second control group consisted of 121 healthy subjects. The participants were considered healthy if they were not regularly taking medication and were not suffering from any diseases known to affect the heart. Data from both the baseline examination of ULSAM at age 50 years and the follow-up examination at age 70 years were used in the analyses.

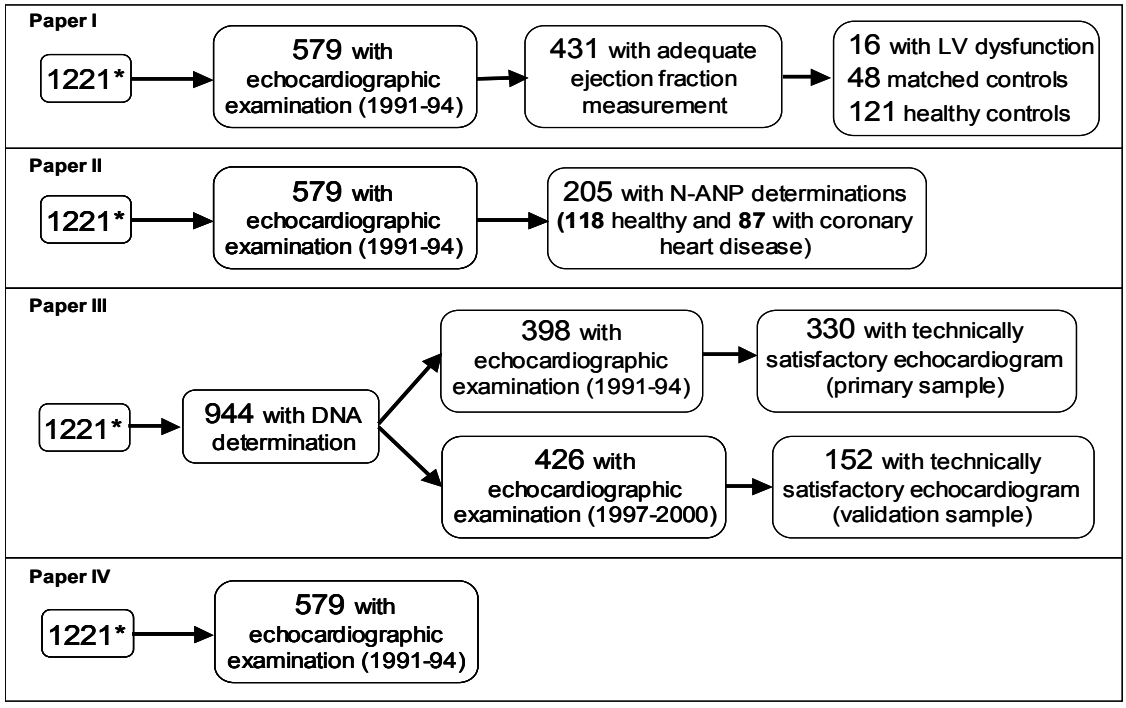


**Figure 2** Number of subjects and inclusion criteria in the study populations of papers I-IV
* All of the study participants at the investigation at age 70 years (1990-94)

**Paper II** N-ANP was analyzed in the men with coronary heart disease (previous myocardial infarction or angina pectoris) and the apparently healthy men from the subgroup of 579 men who had an echocardiographic examination (1991-94) (n=205). These men comprise the study population of paper II.

**Paper III** 944 out of the 1221 men at age 70 years had DNA determinations. Of these men, 330 from the echocardiographic examinations in 1991-94 (primary sample) and 152 from the echocardiographic examination in 1997-2000 (validation sample) had technically satisfactory echocardiogram to assess main indicators of left ventricular function and geometry.

**Paper IV** Paper IV was performed in the 579 men who had an echocardiographic examination at age 70 years (1991-94).

## Investigations at age 50 years

Data from the investigations at age 50 years were only used in Paper I. As previously described[53], the examinations performed included blood sampling and measurements of blood pressure, and anthropometrics.

*Anthropometrics*

Height (without shoes) was measured to the nearest whole cm and weight (in under shorts) to the nearest whole kg. Body mass index (BMI) was calculated as the ratio of the weight to the height squared ($kg/m^2$).

*Blood pressure*

Blood pressure was measured on the subjects' right arm, in the recumbent position after 10 minutes' rest, using a mercury manometer. The heart rate data was taken from an electrocardiogram recording.

*Blood sampling*

Blood sampling with determination of glucose, insulin, cholesterol, HDL, triglycerides and electrolytes was drawn in the morning after an overnight fast. LDL cholesterol was calculated using Friedewald's formula: LDL=serum cholesterol-HDL-(0.42·serum triglycerides). The fatty acid composition of the serum cholesterol esters (14:0, 16:0, 16:1, 18:0, 18:1, 18:2 n6, 18:3 n6, 18:3 n3, 20:3 n6, 20:4 n6, 20:5 n3, 22:6 n3) was determined by gas liquid chromatography[54]. The concentrations of intact and 32-33 split proinsulin were analyzed in 1995-98 by a two-site immunometric assay technique[55], in Cambridge, United Kingdom, in plasma samples that had been stored frozen (-70° C) since baseline. Due to a freezer failure, proinsulin-like molecules were determined in baseline plasma samples from 208 of the subjects comprised in Paper I.

## Investigations at age 70 years

The cohort was reinvestigated twenty years later with an echocardiographic and Doppler examination, 24-hour ambulatory blood pressure monitoring, euglycaemic hyperinsulinaemic clamp, N-ANP determinations and DNA determinations in addition to the study protocol at age 50 years[56]. Anthropometrics, office blood pressure and blood sampling with determinations of the fatty acid composition of the serum cholesterol esters and proinsulin was performed as described at age 50 years.

## Echocardiography

Two-dimensional (2D) echocardiography and Doppler examination was performed with a 2.5 MHz transducer (Sonos 1500, Hewlett Packard Andover, Mass. USA). All examinations were performed with the subjects in the standard left lateral position and in expiratory apnea or quiet breathing. All measurements were obtained on-line and stored in the computer of the ultrasonic unit for later printout. An experienced physician (B.A.) did both the examination and the reading of the images, unaware of the clinical data of the subjects.

Left ventricular dimensions were measured with M-mode using a leading edge-to-edge convention. The measurements included intraventricular septal thickness (IVS), posterior wall thickness (PW), left ventricular diameter in end of diastole and end of systole (LVEDD and LVESD) and left atrial dimension. Left ventricular relative wall thickness, was calculated as (IVS+PW)/LVEDD. Left ventricular volumes were calculated according to the Teichholz M-mode formula (volume=$7D^3/(2.4+D)$, D=diameter)[57,58] and from that, ejection fraction was calculated ((LVEDD - LVESD)/LVEDD).

Left ventricular mass was determined by using the M-mode formula of Troy according to the recommendations by American Society of Echocardiography[59]. To correct for differences in body constitution, left ventricular mass was divided with body surface area giving left ventricular mass index.

Using the upper normal limit for left ventricular mass index of 150 g $m^{-2}$ and a partition value of 0.44 for relative wall thickness according to Ganau et al.[60], the left ventricles were classified into four geometric groups: normal, concentric remodeling, eccentric left ventricular hypertrophy and concentric left ventricular hypertrophy. Left ventricular geometry was considered normal if relative wall thickness was < 0.44 and left ventricular mass index < 150 g $m^{-2}$. A normal mass with increased relative wall thickness was designated left ventricular concentric remodeling according to Ganau et al.[60], while a hypertrophied left ventricle was denoted eccentric if the relative wall thickness was normal, and concentric if relative wall thickness was increased.

Left ventricular atrio-ventricular plane displacement was measured by M-mode at the lateral side of the mitral annulus (performed in only the last 218 consecutive subjects the patients investigated with echocardiography 1991-94). The systolic 2D-index was calculated as: atrio-ventricular plane displacement + (5/LVESD)[61].

Left ventricular wall motion score was calculated as the mean score in a 16-segment model of the left ventricle using 2D images, in which each segment was given a score in the range 1 to 4, using 1=normal wall motion, 2=hypo kinetic wall motion, 3=akinetic and 4=paradoxical wall motion (dyskinesi)[62]. In the statistical analyses, left ventricular wall motion score was dichotomized in to two groups in Paper IV (left ventricular wall motion score=1 or >1).

Pulsed Doppler from the apical position was used to measure left ventricular inflow through the mitral valve. The peak velocities of the early rapid filling (E-wave) and filling during atrial systole (A-wave) were recorded and the E/A-ratio was calculated. The deceleration time was measured as the interval between the peak of the E-wave and the point at which the descending segment of the E-wave or its asymptote crosses the zero velocity line. Left ventricular isovolumic relaxation time was measured as the interval between aortic valve closure and the onset of mitral flow. In order to treat the E/A-ratio as a continuous variable, subjects with a restrictive pattern (E/A-ratio > 2.5) or with suspected 'pseudonormalization' (left ventricular systolic dysfunction with E/A-ratio > 1.2) were excluded from the analysis (n=9) using E/A-ratio and isovolumic relaxation time as continuous variables.

Left ventricular ejection time (ET) was measured from the onset to the end of left ventricular outflow velocity pattern. The mitral closing-to-opening time (a) was measured as the interval from the end to the onset of the mitral inflow velocity pattern. Mean values of three measurements were used and the myocardial performance index was calculated as (a-ET)/ET[16].

Left ventricular outflow tract (LVOT) diameter was obtained from a parasternal long axis view, while the flow velocity integral (FVI) was determined from the apical with the Doppler sample volume at approximately the same level as the diameter measurement was obtained. From these two variables stroke volume was calculated ((p*$LVOT^2$)/(4*FVI). Cardiac output was calculated as stroke volume*heart rate. The stroke index was obtained by dividing stroke volume with body surface area and the cardiac index was obtained by dividing cardiac output with body surface area.

A complete repeat investigation was performed in 22 randomly selected subjects approximately one month after the initial investigation. Intra-class-correlation coefficients for the M-Mode measurements: left ventricular mass index 0.65 and ejection fraction 0.52. For the Doppler measurements the intra-class-correlation coefficients were as follows: E/A-ratio 0.72, isovolumic relax-

ation time 0.84, stroke volume 0.82, cardiac output 0.74, deceleration time 0.71, Myocardial Performance Index 0.66. The kappa-value for left ventricular wall motion score index (considered a dichotomous variable in Paper IV) was 1[63].

## Euglycaemic hyperinsulinaemic clamp

Euglycaemic hyperinsulinaemic clamp technique according to DeFronzo[64] was used to estimate in vivo sensitivity to insulin. Insulin (Actrapid Human(r), Novo, Copenhagen, Denmark) was infused in a primary dose for the first 10 minutes and then as a continuous infusion (56 mU/min/body surface area ($m^2$)) for two hours. The glucose infusion rate during the last hour was used as a measure of insulin sensitivity (M-value).

## N-ANP measurement

A Delfia sandwich immunoassay for measurements of N-ANP was developed[65]. One monoclonal antibody (Medix Biochemica OY, Kauniainen, Finland), directed against amino acids 1-30 in proANP, was used as catcher antibody and another monoclonal antibody (Medix), directed against amino acids 79-98, was used as detector antibody. The catcher antibody was biotinylated by standard methods. The detector antibody was labeled with Europium (Eu) according to purchaser instructions (Delfia Eu-labeling kit, Wallac OY, Turku, Finland). The incubation was performed in 96-wells micro titer plates pre-coated with strepavidin. For dilution of antibodies, a standard Delfia assay buffer was used (hTSH-ultra buffer, Wallac). Pooled patient serum was used as standards. Initial standardization was performed against a pooled serum preparation kindly provided by Medix Biochemical. Dilution of standards was made in serum pre-treated with active charcoal to remove interfering peptides.

Serum samples and standards were assayed pre-diluted 1:4 with assay buffer and dispensed into micro titer wells (100 µL). Pre-diluted catcher-antibody and detector-antibody, 50 µL each, were added and the micro titer plates were incubated in room temperature for 2 hours on a shake board. After washing, 200 µL enhancement solution was added and the plates were analyzed in a Delfia fluorometer. Calculation of standard curve and patient results was performed on a MultiCalc program.

Assay performance, given as within-assay variation, was 8%, 4% and 6% at serum levels of 140, 970 and 3500 pmol/l respectively. The total inter-assay variation was 12%, 10% and 9% respectively at these serum concentrations.

The reference range for N-ANP in healthy blood donors older than 40 years of age was calculated to <550 pmol/l [65]. The withdrawal conditions for the blood samples were standardized and performed in the morning on subjects in a fast-

ing state. The N-ANP samples were withdrawn a couple of months before the echocardiographic examination. The blood samples had been stored at minus 70 degrees Celsius for 3-5 years prior to N-ANP level determination.

## Genetic analyses

*SNP discovery*

To identify polymorph positions in the hUNC-93B1 gene, twenty-two fragments covering selected regions of the gene were amplified using genomic DNA from 15-30 unrelated anonymous individuals. GenBank accession number AC004923 covers the genomic sequence of the hUNC-93B1 gene and was selected as master sequence. The 3'-end of the gene was avoided, since that region is extremely homologous to other sequences in the human genome and there is risk of unspecific amplification. After amplification, detection of genetic variation in the hUNC-93B1 gene was performed using solid phase sequencing (AutoLoad™ Solid-Phase Sequencing kit, Amersham Pharmacia Biotech) and gel electrophoresis on ALFexpress™ sequencers (Amersham Pharmacia Biotech).

*SNP typing*

Fragments covering each of the selected SNPs were amplified from genomic DNA from the 330 individuals in the primary sample. Detection of SNPs using the PSQ platform (Pyrosequencing AB) was performed according to the manufacturers instructions. PSQ sample preparation VP93, VP99, VP101 and VP102: Capture at 60°C for 30 min and annealing at 80°C for 2 min. PSQ sample preparation VP94: Capture at 60°C for 30 min and annealing at 95°C for 2 min.

*PCR components and conditions*

Total reaction volume was 50µl: GeneAmp®10X PCR-buffer II, 1.5 mM MgCl2 (Perkin Elmer), 0.125 mM dNTP (Ultrapure dNTP-set purchased from Amersham Pharmacia Biotech), 0.2µM of each primer, (Scandinavian Gene Synthesis), 0.65 U AmpliTaqGold™ DNA polymerase (5U/µl) (Perkin Elmer), and 0.2 ng/µl of DNA-sample. Amplification was performed using a GeneAmp™ PCR Systems 9700 from Perkin Elmer and the following conditions: 95°C 10 min 45 x (95°C 30 s, Ta 60°C 45 s, 72°C 45 s) 72°C 5 min, 22°C.

*Haplotype analysis*

The analysis was performed in the primary sample using Haplotype Resolver, which is a software based on the maximum likelihood methodology, and use of the EM algorithm[66].

In order to detect miss-genotyping, Hardy-Weinberg equilibrium, haplotype analysis and duplicate control were performed. No deviation in genotyping result could be seen in the 100 duplicates checked. The frequency of the three different genotypes for each SNP did not differ significantly from expected values in the Hardy-Weinberg calculations. In the haplotype analysis, two of the detected haplotypes appeared only once. As this was probably the result of an incorrect genotype calling, they were not considered true haplotypes. Haplotype analysis could be performed on a total of 310 individuals.

## Ambulatory blood pressure measurements

The ambulatory blood pressure measuring device Accutracker II (Suntech Medical Instruments, Raleigh, NC) was attached to the subjects' non-dominant arm by a skilled lab technician. Systolic and diastolic blood pressures were measured every 30 min during daytime (0600-2300) and every hour during nighttime over 24 hours.

## Definition of Cardiovascular Risk Factors

Traditional cardiovascular risk factors were selected and defined as follows: hypertension (systolic blood pressure >160mm Hg and/or diastolic blood pressure >95 mm Hg and/or anti-hypertensive medication), hyperlipidemia (serum cholesterol>6.5 mmol/liter and/or serum triglycerides >2.3 mmol/l and/or lipid lowering medication), diabetes (blood glucose (6.7mmol/l (fasting) and/or (10.0 mmol/l (2-hour oral glucose tolerance test value) and/or anti-diabetic medication), left ventricular hypertrophy (left ventricular mass index >150 g/m2), smoking (from interview reports) and previous myocardial infarction (hospital discharge record).

## Follow-up after investigation at age 70 years

The subjects in paper IV had a mean follow-up time of 6.8 years (range 0.7 to 8.4 years), contributing to 3950 person-years. End-points where defined using the Swedish national cause-of death register. During follow-up, 96 subjects died (rate 2.4/100 person-years at risk (PYAR)); 42 deaths were from cardiovascular disease (ICD10 codes I20 to I79), rate 1.1 /100 PYAR).

## Statistical analyses

Data are given as means ± standard deviations. Logarithmic transformation was performed when W (according to Shapiro-Wilk's test) was <0.95. ANOVA was used to calculate overall differences between groups. Post-hoc analysis of differences between specific groups was only performed if the overall F-test was significant. Pearson's partial correlation coefficient was used to assess relation-

ships between continuous variables. When a normal distribution was not obtainable, Kruskal-Wallis or Spearman-Rank test was performed. Two-tailed 95 % confidence intervals and p values were given, with $p < 0.05$ regarded as significant.

Receiver-operator-characteristic (ROC) curve was calculated from data obtained from logistic regression[67] in paper II.

In order to control the probability for type 1 error in the primary endpoint analyses in the primary sample in Paper III, the statistical significance of the results was ascertained with a permutation test[68]. This procedure holds the probability for type 1 error fixed at 5 % over all tests considered. A p-value from the non-permuted data ($p < 0.000715$), which was lower than the 5th percentile in the permuted distribution, was considered statistically significant.

In the validation sample in Paper III, our primary hypothesis was to validate the relation between the E/A-ratio and the five SNPs and haplotype H3 found in the primary sample. For each combination of the E/A-ratio and the six genotypes, an analysis of variance model was estimated where the genotype was the factor. Using the permutation test, a non-permuted $p<0.019$ was considered statistically significant.

In Paper IV, the prognostic value of one standard deviation increase in the continuous variables, or transfer from one level to another for the dichotomous variable, was investigated with Cox proportional hazard ratios. Adjustments were made for the six above-mentioned cardiovascular risk factors in multivariate Cox proportional hazard analyses. A multivariate Cox proportional hazard ratio analysis was also performed including all of the measurements that were independent predictors of cardiovascular mortality in the univariate analyses. Test of the joint effect of a group of variables was performed with a likelihood ratio test. Kaplan-Meier plots were used to describe survival over time.

The statistical programs JMP 3.2 (SAS Institute Inc., Cary, NC, USA) and STATA 6.0 (Stata corporation) were used to perform the analyses.

# Results and discussion, paper I-IV

**Paper I:** Several factors associated with the insulin resistance syndrome are predictors of left ventricular systolic dysfunction in a male population after twenty years of follow-up.

## Results

*Age 50 years*

Heart rate, fasting glucose, and the proportion of oleic acid (18:1 n 9) and linoleic acid (18:2 n6) in the serum cholesterol esters at age 50 years was significantly correlated to ejection fraction at age 70 years (figure 3) but only heart rate and fasting glucose concentrations were still significantly inversely correlated to ejection fraction after adjustment for hospitalization of acute myocardial infarction, prevalence of hypertension and diabetes, smoking status and treatment with vasoactive drugs.

In the nested case-control analysis, heart rate, serum concentration of proinsulin and the proportion of dihomogammalinolenic acid (20:3 n6) in serum cholesterol esters were significantly higher and the serum concentration of phosphate was significantly lower at age 50 years in the subjects who had left ventricular systolic dysfunction at age 70 years compared to both control groups (figure 4). The prevalence of myocardial infarction, hypertension, diabetes or smoking in the left ventricular systolic dysfunction group and the control groups did not differ between the groups at age 50 years.

*Age 70 years*

Both plasma split proinsulin 32-33 and office heart rate at age 70 years was significantly correlated to ejection fraction (figure 3). However, office heart rate was the only variable significantly inversely correlated to ejection fraction after adjustment for hospitalization of acute myocardial infarction, prevalence of hypertension and diabetes, smoking status and treatment with vasoactive drugs.

No major metabolic abnormalities were associated with left ventricular systolic dysfunction when compared to controls in the cross-sectional case control analyses at age 70 years.

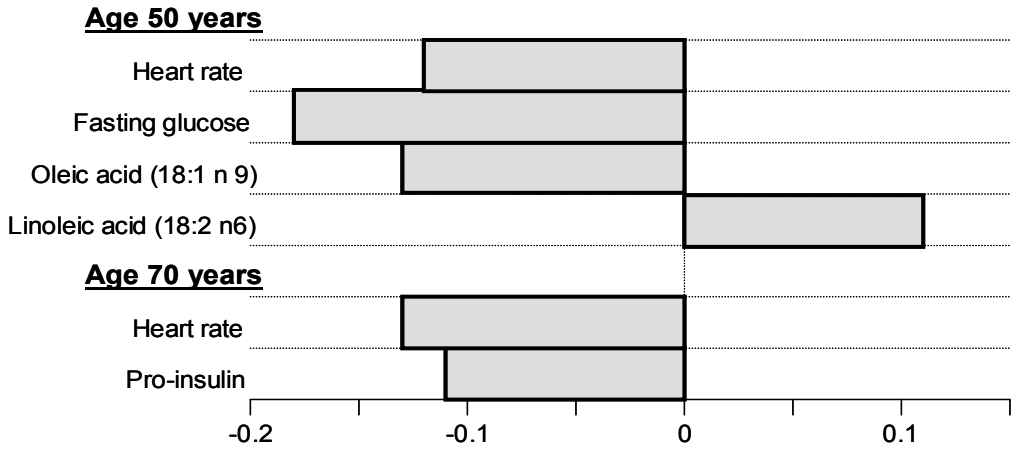


**Figure 3** Significant correlation coefficients between variables at age 50 and age 70, respectively, and ejection fraction at age 70

## Discussion

In the prospective part of this study, we demonstrated that factors associated with insulin resistance[54,69-71], such as an increased heart rate, increased serum concentrations of proinsulin, a high proportion of dihomogammalinolenic acid in serum cholesterol esters and hypophosphatemia, precede left ventricular systolic dysfunction independently of cardiovascular diseases and medication after twenty years follow-up. Furthermore, ejection fraction at age 70 years was inversely correlated to heart rate, glucose concentrations and the proportion of oleic acid (18:1 n9) in serum cholesterol esters at age 50 years and positively correlated with the proportion of linoleic acid (18:2 n6) in serum cholesterol esters at age 50 years, variables also related to insulin resistance[26,54,69].

Our findings raise questions as to whether the differences seen in the left ventricular dysfunction group compared to the disease matched controls at age 50 years merely reflect a discrepancy between the groups in disease duration/severity or if it indicates a different disease process leading to left ventricular systolic dysfunction. The fact that tachycardia and increased serum concentrations of proinsulin and a high proportion of dihomogammalinolenic acid in

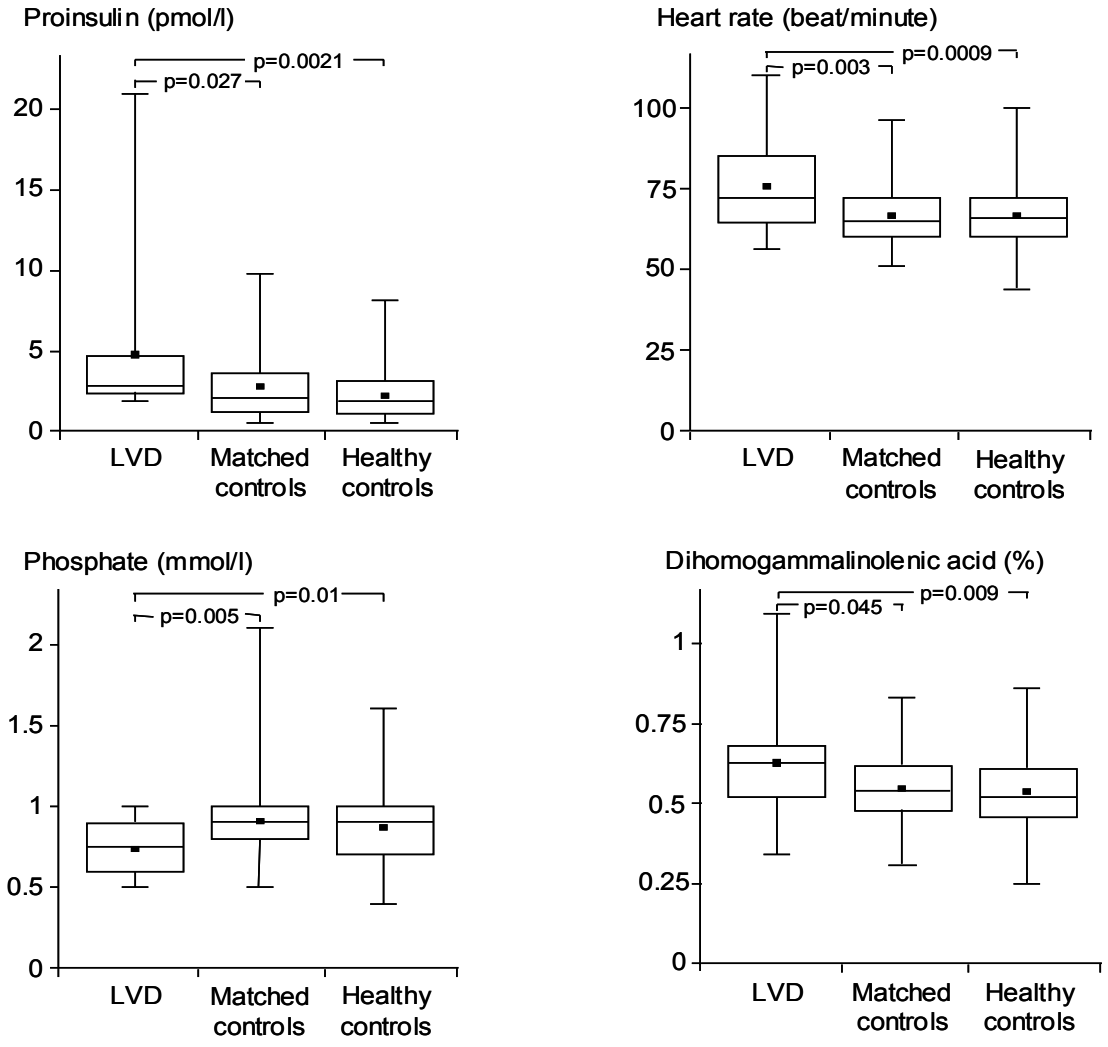


**Figure 4** Plasma proinsulin concentrations, heart rate, serum phosphate concentrations and proportion of dihomogammalinolenic acid (20:3) in serum cholesterol esters at age 50 in subjects with left ventricular systolic dysfunction (LVD), in controls matched for cardiovascular disease, diabetes and cardiovascular medication and in healthy controls, defined at age 70. The lowest, second lowest, middle, second highest and highest boxpoints represent the minimum, 25th percentile, median, 75th percentile and maximum respectively. Means indicated by filled square.

serum cholesterol esters have all been shown to be independent risk factors for cardiovascular morbidity and mortality[69,72,73] is in support of the former view. Moreover, there seem to be a direct atherogenic action of an increased heart rate due to blood flow characteristics[74] and the proinsulin-molecule may also play a direct role in the development of cardiovascular disease, as clinical trials comparing treatment with proinsulin to insulin treatment in diabetic patients were prematurely terminated due to an increase in myocardial infarctions in subjects treated with proinsulin[75]. Phosphate concentrations decrease after infusion of epinephrine[76] and an inverse correlation between phosphate concentrations and insulin concentrations has previously been shown in this population[77]. The low phosphate together with the increased heart rate may point toward an association between an enhanced sympathetic tone with markers of insulin resistance in the left ventricular dysfunction group.

Supporting the hypothesis that there may be an alternative disease process involved is the fact that insulin resistant patients have an altered structure of the myocardium with interstitial fibrosis and a reduced coronary blood flow reserve, leading to myocardial stiffness and impaired left ventricular filling[78]. These morphological and functional changes could be an important factor in reducing the ability of the myocardium to adapt to hypertension and/or a myocardial infarction, and there was a tendency towards an impaired diastolic function (low E/A-ratio, p=0.07) in the left ventricular systolic dysfunction group (data not shown). The correlation of oleic acid (18:1 n9) and linoleic acid (18:2 n6) in serum cholesterol esters at age 50 years to ejection fraction at age 70 years, in addition to the increased proportion of dihomogammalinolenic acid in the left ventricular systolic dysfunction group, may indicate that dietary factors play a part in the development of left ventricular systolic dysfunction. The fatty acid composition could also be secondary to genetic variations in the activities of enzyme regulating desaturation and elongation of fatty acids in the body.

Based on prospective data, we conclude that several components associated with the insulin resistance syndrome are predictors of left ventricular systolic dysfunction after twenty years in a male population sample.

# Paper II: N-terminal atrial natriuretic peptide and left ventricular geometry and function in a population sample of elderly males

## Results

The subjects with coronary heart disease had higher plasma levels of N-ANP than the healthy group (p=0.003). However, when excluding the subjects with an ejection fraction ≤ 0.40, the occurrence of coronary heart disease was no longer significantly associated with elevated N-ANP levels (p=0.09) while the N-ANP levels were significantly increased in the left ventricular dysfunction group compared to the group of healthy subjects (p<0.0001, figure 5).

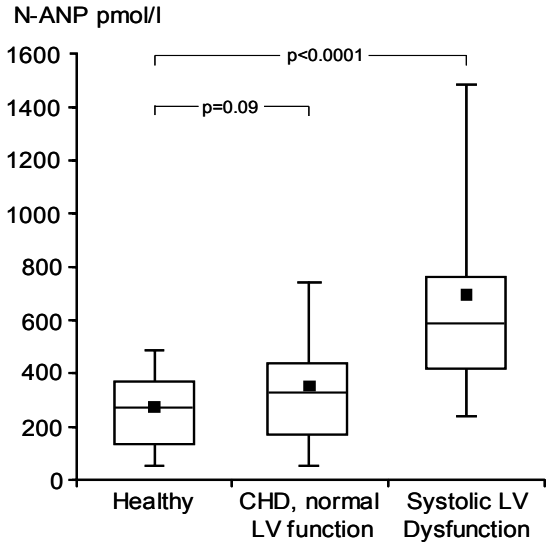


**Figure 5** Plasma levels of N-terminal natriuretic peptide (N-ANP) in 102 healthy subjects, 67 subjects with coronary heart disease (CHD) without systolic left ventricular (LV) dysfunction and 14 subjects with both Coronary Heart Disease and systolic LV dysfunction. The lowest, second lowest, middle, second highest and highest boxpoints represent the 10th percentile, 25th percentile, median, 75th percentile and 90th percentile respectively. Means indicated by filled square.

As shown in figure 5, there is a great overlap between healthy subjects and those with left ventricular dysfunction. The area under the receiver-operating characteristic (ROC) curve for N-ANP, as a test for left ventricular dysfunction, was found to be 0.83 (p<0.0001, figure 6). Using a plasma level of 398 pmol/l for N-ANP as the cut-off level, the sensitivity was found to be 0.86 and the specificity was 0.75. A cut-off level for plasma N-ANP of 550 pmol/l gave a sensitivity of 0.57 and a specificity of 0.91.

N-ANP levels differed significantly between the left ventricular geometric groups (p=0.022). The highest levels of N-ANP were observed in the two groups with left ventricular hypertrophy. However, post hoc analysis showed

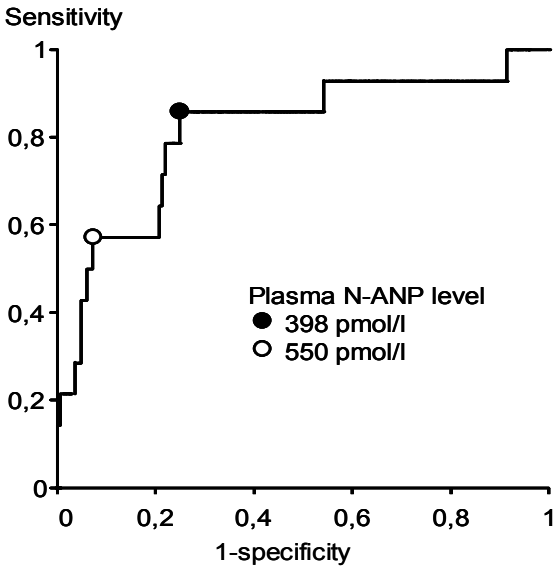


**Figure 6** Receiver-operating-characteristic curve for ability of N-ANP to detect systolic left ventricular dysfunction in the study population. Proposed cut-off values of plasma levels of N-ANP indicated.

only the difference between the normal and the eccentric hypertrophy to be statistically significant (p=0.0035). When further dividing the subjects into subjects with an ejection fraction > 0.40 and subjects with an ejection fraction ≤ 0.40 (figure 7), subjects with a normal ejection fraction in the eccentric hypertrophy group showed similar levels of N-ANP as the corresponding subjects in the normal group.

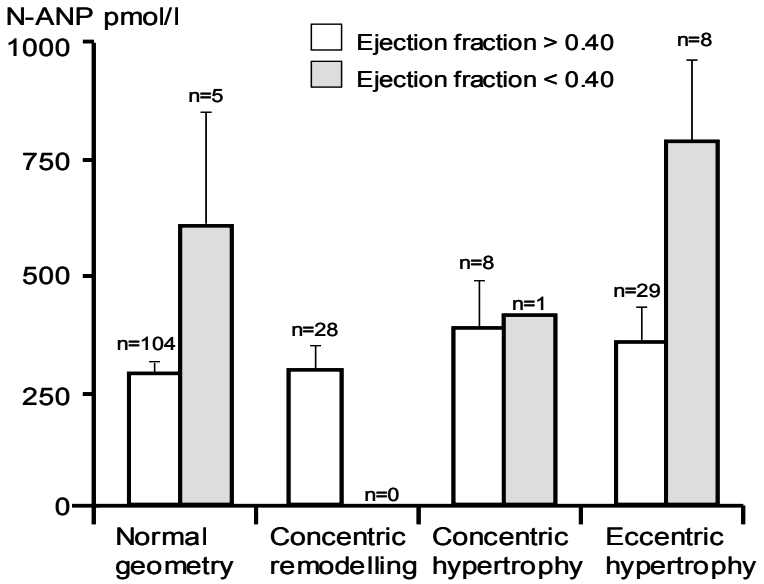


**Figure 7** N-ANP levels in subjects with or without left ventricular dysfunction according to left ventricular geometry. Standard error of the mean indicated. n=number of subjects

## Discussion

Natriuretic peptides have been suggested as a screening tool in the diagnosis of congestive heart failure[38,39,44,79], and detecting patients with symptomatic and asymptomatic left ventricular systolic dysfunction at an early stage is crucial in order to lower the morbidity and mortality associated with this disease. In accordance with other investigators[38,40,42-44,79], elevated levels of N-ANP were found in subjects with left ventricular dysfunction. However, based on the former studies and the findings in the present study, the sensitivity and specificity of N-ANP as a diagnostic test for congestive heart failure seem limited. Using a N-ANP plasma level of 398 pmol/l as the cut-off level, the sensitivity was found to be 0.86 and the specificity was 0.75. In our opinion, the sensitivity is too low to use this as a routine method of determining left ventricular dysfunction. Increasing the cut-off level for plasma N-ANP to 550 pmol/l gave a sensitivity of 0.57 and a specificity of 0.91. This approach might be useful in routine care when selecting patients suitable for echocardiographic examination in order to detect asymptomatic left ventricular dysfunction.

In accordance with a previous study by Nishikimi and colleagues, measuring ANP levels in a population of hypertensive subjects[80], the present study showed elevated levels of N-ANP in subjects with left ventricular hypertrophy. In the previous study the highest levels of ANP were seen in the concentric left ventricular hypertrophy group, while in the present study the highest N-ANP levels were observed in the subjects with eccentric left ventricular hypertrophy. However, as only nine subjects in the present study showed concentric left ventricular hypertrophy, the results in this subgroup should be taken with caution. Another important difference between the results in the two studies is that no difference in left ventricular systolic function, based on the fraction shortening, between the left ventricular geometric subgroups was seen in the former study[80], while several subjects with systolic dysfunction were found in the group with eccentric left ventricular hypertrophy in the present study. The importance of this is shown in figure 7, where subjects with eccentric left ventricular hypertrophy and a normal ejection fraction showed similar N-ANP levels as subjects with normal left ventricular geometry and a normal ejection fraction. Thus, the association between the occurrence of eccentric left ventricular hypertrophy and elevated N-ANP levels is most likely due to a high proportion of subjects with left ventricular dysfunction in the group with eccentric left ventricular hypertrophy.

In conclusion, plasma N-ANP levels were significantly increased in subjects with left ventricular dysfunction in comparison to healthy subjects in this population-based sample of elderly males. However, the diagnostic accuracy was poor due to the extensive overlapping between the groups. Furthermore, this study showed that elevated N-ANP levels in subjects with left ventricular hypertrophy might in part be explained by a low ejection fraction in subjects with eccentric hypertrophy.

## **Paper III:** hUNC-93B1, a novel gene mainly expressed in the heart, is related to left ventricular diastolic function in elderly men.

## Results

*Genotyping: SNP discovery and haplotype typing*

Six SNP's and six haplotypes were identified. The five most common SNP's and the four most common haplotypes were included in the association study.

*Phenotype association*

**Primary sample** The genotype homozygous for the haplotype H3 showed higher level of E/A-ratio compared to non-carriers after permutation test and adjustment for myocardial infarction, blood pressure, heart rate, BMI, diabetes and the use of cardiovascular medication (figure 8). The relations between the E/A-ratio and the genotypes vp93, vp94, vp101 and vp102 did not reach statistical significance after accounting for the multiple testing using the permutation test. No relations were seen between the genotypes H1, H2, or H4 and the E/A-ratio or between any of the other primary phenotype variables (ejection fraction, left ventricular mass index, relative wall thickness and QT-dispersion).

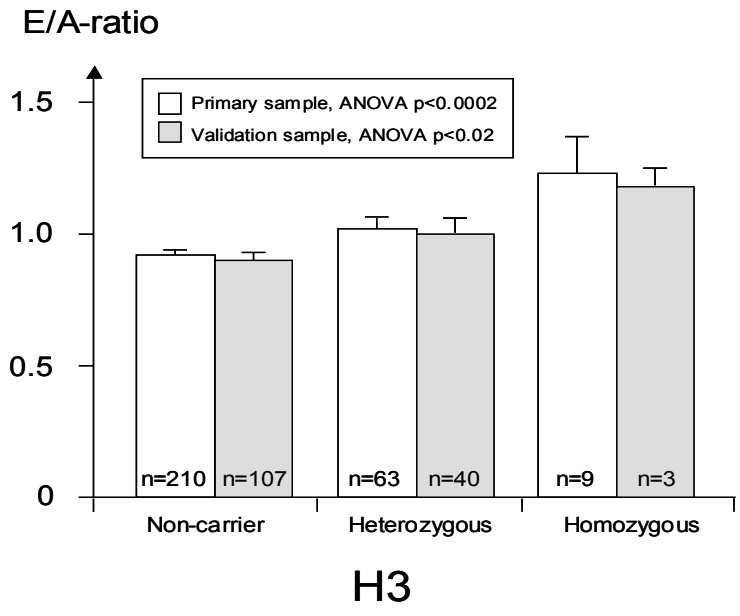


**Figure 8** Relation between the genotypes of haplotype H3 of the hUNC-93B1 gene and left ventricular diastolic function in the primary sample and the validation sample. Data are adjusted for myocardial infarction, blood pressure, BMI, diabetes, heart rate and the use of cardiovascular medication. Standard error of the mean indicated.

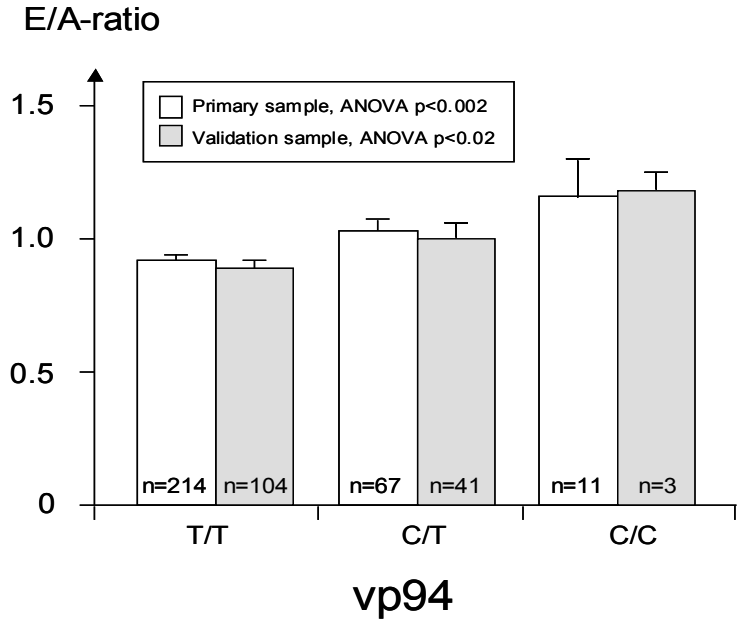


**Figure 9** Relation between the genotypes of the SNP vp94 of the hUNC-93B1 gene and left ventricular diastolic function in the primary sample and the validation sample. Data are adjusted for myocardial infarction, blood pressure, BMI, diabetes, heart rate and the use of cardiovascular medication. Standard error of the mean indicated.

**Validation sample** The genotype homozygous for H3 had higher level of E/A-ratio compared to non-carriers and the C/C allele of vp94 had higher level of the E/A-ratio compared to the T/T allele after permutation test and adjustment for myocardial infarction, blood pressure, heart rate, BMI, diabetes and the use of cardiovascular medication (figure 8 and 9).

## Discussion

As the novel hUNC-93B1 gene has the highest expression in the heart we wanted to investigate the relation between different hUNC-93B1 genotypes and cardiac phenotypes. In the first phase of the study we identified the most common SNPs and haplotypes. In the second phase, five primary end-point variables were chosen to reflect different aspects of cardiac function. A relation between haplotype H3 of the hUNC-93B1 gene and the E/A-ratio was found. As a third phase, in order to further strengthen the findings, we validated the results in another, slightly older, sample of the same cohort.

The E/A-ratio, one of the most widely used measurements of diastolic function, is affected by several cardiovascular risk factors like age, previous myocardial infarction, diabetes, hypertension, heart rate, obesity and the use of cardiovascular medication (Ärnlöv, unpublished data)[81]. That the present associations between genotypes of hUNC-93B1 and the E/A-ratio were independent of these factors suggest that there may be alternative mechanisms that mediate the effects of the gene.

The physiology of diastolic function is characterized by complex interactions between left atrial and left ventricular pressures, left ventricular cellular derangements, and myocardial relaxation and compliance. From the echocardiographic view, left ventricular diastolic function could be divided into three distinct parts. The isovolumic relaxation time is the earliest part of diastole

and related to removal of $Ca^{2+}$ from the cytoplasm by $Ca^{2+}$-ATPases. Thereafter the transmitral filling of the left ventricle begins giving rise to the E-wave measured by Doppler. After this early filling period of the left ventricle, the flow into the left ventricle is further enhanced by the atrial contraction, giving rise to the A-wave. The relationship between the early and atrial filling periods is quantified by the E/A-ratio. Several factors may influence this ratio, but a reduced compliance of the left ventricle is suggested to be one of the major factors determining a reduced E/A-ratio. Such a reduction in left ventricular compliance could either be due to structural changes, as typically seen in left ventricular hypertrophy or functional, as seen in the ischaemic myocardium or during stunning. With the limited knowledge of the action of the hUNC-93B1 gene it seems most likely that the gene would influence the functional part of left ventricular compliance.

Functionally abnormal variants of the UNC-93 gene in C. Elegans result in uncoordinated muscle activity[82] and Levin et al. speculated that UNC-93 either disrupt communication between muscle cells by influencing gap junction or act in the response of muscle cells to excitation-contraction coupling[52]. These putative effects in C. Elegans may be compatible with an effect on myocardial performance characteristics and provide an explanation for the observations in the present study. The fact that the findings were independent of myocardial infarction supports a hypothesis of a direct myocardial effect of the gene, which is not mediated by coronary atherosclerosis. However, it should be pointed out that this study does not tell anything about the role or function of the gene, which will be a task for further molecular genetic studies, as well as for further studies of phenotype characteristics.

As haplotypes are a combination of different SNPs, they are suitable in association studies. A haplotype may be able to reveal more information about the genotype/phenotype association than a single SNP. In this study, the subjects with a C/C-allele of vp94 were more or less the same as the subjects homozygous for haplotype H3, thus the haplotype did not portray more information than the single SNP.

In an association study like this we cannot distinguish between whether the link between hUNC-93B1 and E/A-ratio is causal, i.e. whether the gene is involved in the pathophysiology of left ventricular diastolic function, or if it is due to linkage disequilibrium with another functional gene. However, the location of vp94 in the putative promoter region implies that this SNP may be located in a functional segment of DNA.

Some of the previous genotype association studies have been performed in small populations or in various subgroups, which may have resulted in statistical artifacts and bias, leading to false positive or false negative conclusions. In this study, we tried to avoid these pitfalls by control for multiple testing, ad-

justment for possible confounders and validation of the findings in a separate group. In our view, these procedures strengthen the validity of our findings considerably.

The cDNA of hUNC-93B1 comprises 2282 base pairs, corresponding to 597 amino acids in the hUNC-93B1 protein. Structure prediction analysis of the protein sequence has identified 2 distinct domains, the NH2 terminal part, which is very hydrophilic, whereas the rest of the protein constitutes a 12 transmembrane domain structure[51]. If the hUNC-93B1 protein is located in the cell membrane it might be a possible target site for future pharmacological intervention. Currently, there is a need for drugs that improve left ventricular diastolic function. Up to half of the patients diagnosed with heart failure have been suggested to have a primary left ventricular diastolic dysfunction[15] and at present, there are only a few randomized clinical trial on treatment of primary diastolic heart failure.

In conclusion, relations between the SNP vp94 and haplotype H3 of the hUNC-93B1 gene and the E/A-ratio were found and validated in a population-based cohort of elderly men. This finding suggests the possibility of a novel mechanism, that in the future could offer new therapeutic opportunities for diastolic heart failure.

## **Paper IV:** A Doppler-derived index of combined left ventricular systolic and diastolic function is a powerful predictor of cardiovascular mortality in elderly men.

### Results

*Univariate analyses*

In univariate Cox proportional hazard analyses for a 1-SD increase, indices reflecting left ventricular systolic function (ejection fraction, left ventricular wall motion score index, atrio-ventricular plane displacement and Systolic 2D-index), left ventricular diastolic function (E/A-ratio) and left ventricular global function (myocardial performance index) were all found to be significant predictors for cardiovascular mortality. When adjusting for six cardiovascular risk factors (previous myocardial infarction, hyperlipidemia, hypertension, diabetes, smoking status and left ventricular hypertrophy), all but left ventricular wall motion score index were still predictors for cardiovascular mortality.

The above predictors for cardiovascular mortality were also significant predictors for total mortality, but with less predictive capacity and wider confidence intervals.

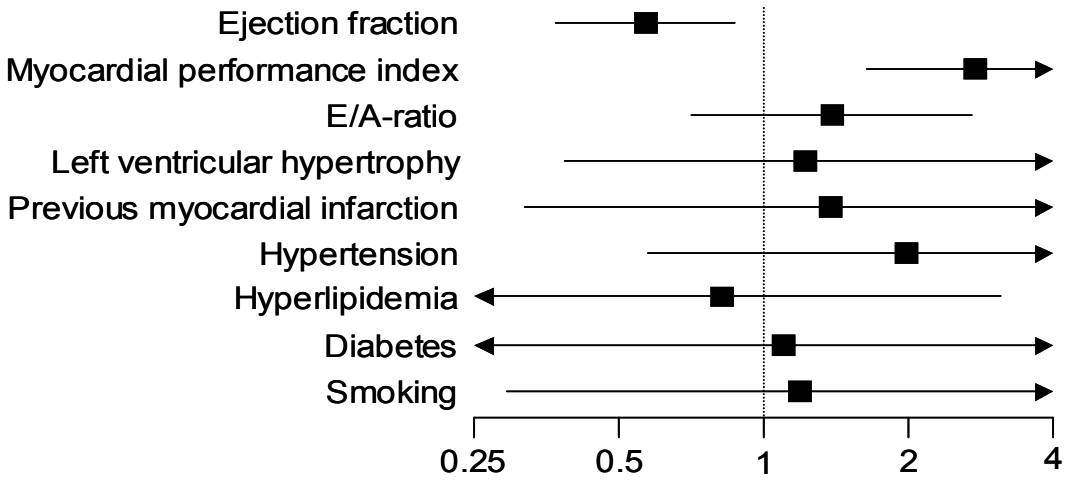


**Figure 10** Multivariate Cox Proportional Hazard analysis for the prediction of cardiovascular mortality. Boxes are point estimates of hazard ratio for 1-standard deviation increase for the continuous variables (ejection fraction, myocardial performance index and E/A-ratio) and occurrence compared to no occurrence for the dichotomous variable. Lines indicate 95 % confidence intervals.

*Comparison between the predictive capacities of myocardial performance index, ejection fraction and E/A-ratio*

In a multivariate Cox proportional hazard ratio model including the most commonly used measurements of systolic and diastolic function (ejection fraction and E/A-ratio) and the novel myocardial performance index together with six traditional cardiovascular risk factors as independent variables, both ejection fraction (HR for 1-SD increase =0.59, 95% CI 0.38-0.90, p<0.05) and myocardial performance index (HR for 1-SD increase =2.92, 95% CI 1.71-4.97, p<0.0001) were found to be independent predictors for cardiovascular mortality (figure 10). Neither E/A-ratio, nor any of the traditional risk factors for cardiovascular mortality remained significant in the model. A test of the traditional risk factors joint effect gives p=0.80 when ejection fraction and myocardial performance index were taken into account. The interaction terms between myocardial performance index and ejection fraction was not a significant predictor in the analysis. When we stratified for previous myocardial infarction, myocardial performance index and ejection fraction were still the only independent predictors when excluding subjects with a previous myocardial infarction (data not shown). In the subjects with a previous myocardial infarction, myocardial performance index was the only significant predictor.

Subjects with both an ejection fraction <0.5 (the lowest 10th percentile) and a myocardial performance index >0.9 (the highest 10th percentile) had almost twenty-five times higher hazard ratio compared to subjects with both normal ejection fraction (>0.5) and normal myocardial performance index (<0.9) (figure 11). Subjects with normal ejection fraction, but with a myocardial performance index >0.9 had almost eleven times higher hazard ratio compared with a normal left ventricular performance. Subjects with an ejection fraction <0.5 but with a normal myocardial performance index did not differ compared to normal subjects. Figure 12 shows the survival over time in the four groups.

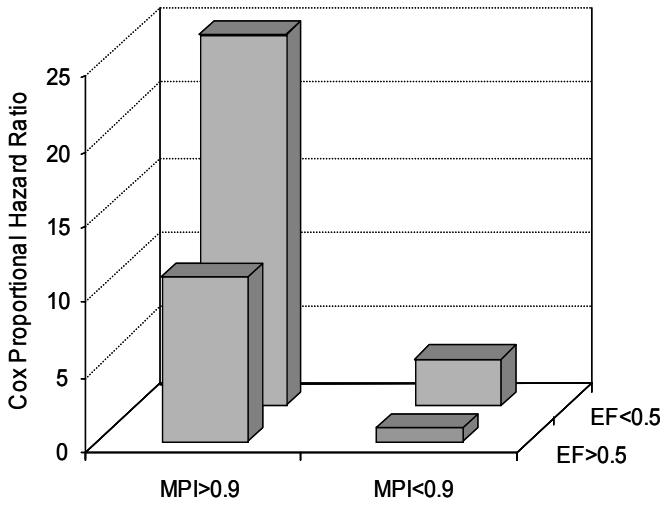


**Figure 11** Bars are Cox proportional Hazard ratios in four groups, ejection fraction <0.5 or >0.5 and Myocardial Performance Index >0.9 or <0.9. EF= ejection fraction, MPI= myocardial performance index

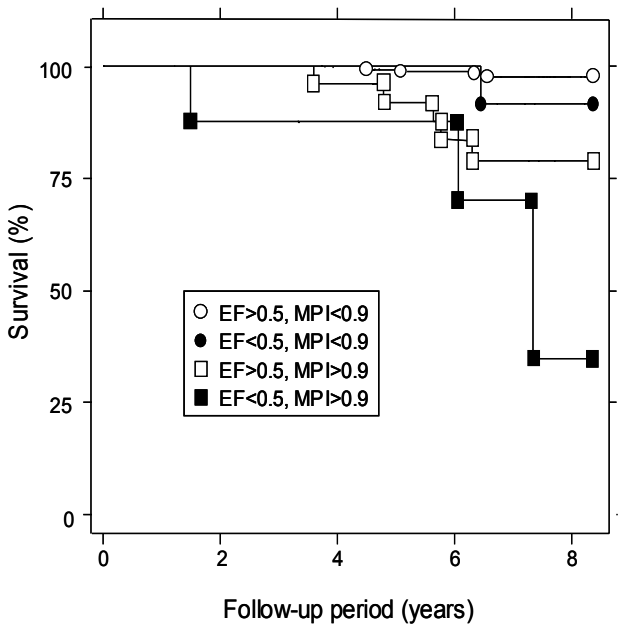


**Figure 12** Kaplan Meier curves showing survival from cardiovascular mortality over the follow-up period. EF= ejection fraction, MPI= myocardial performance index.

*E/A-ratio, pseudo-normalization and restrictive pattern*

When using E/A-ratio in the above analyses we excluded subjects with restrictive or pseudo-normalization pattern (n=9) in order to treat E/A-ratio as a continuous variable. When dividing E/A-ratio into three groups (normal E/A ratio (E/A >0.65), low E/A-ratio (E/A < 0.65) and pseudo-normalization or restrictive pattern), the subjects with low E/A had an increased risk for cardiovascular mortality compared to the subjects with normal E/A even after adjustment for the above-mentioned cardiovascular risk factors (multivariate hazard ratio=3.8, $p<0.005$). There was no difference between the pseudo-normalized/restrictive group and the group with normal E/A.

## Discussion

In a multivariate analysis using indices reflecting systolic function (ejection fraction), diastolic function (E/A-ratio) and cardiac global function (myocardial performance index) both myocardial performance index and ejection fraction were found to be predictors for cardiovascular mortality independent of each other and of traditional cardiovascular risk factors (figure 10). In fact, none of these well-established cardiovascular risk factors remained significant in the model, neither separately nor as a group of variables. This is worth mentioning, as the same result was found also when excluding subjects with a previous myocardial infarction, i.e. in a primary preventive setting. In clinical practice, a great deal of effort and resources are put into identifying patients at high risk for cardiovascular disease and mortality. This study raises the question if an echocardiographic examination should be included in a primary preventive assessment of cardiovascular risk. After taking myocardial performance index and ejection fraction into account, the traditional cardiovascular

risk factors did not provide any additional major independent prognostic information. It should be pointed out that further studies are needed; one should always be cautious about applying a clinical decision rule developed in one population to another without first assessing its accuracy in that population[83]. Furthermore, it has not been established whether subjects with high myocardial performance index benefits from pharmacological intervention and additional studies are needed which address the issue of cost-effectiveness in using an echocardiographic examination as a screening tool when trying to identify patients at high risk for cardiovascular mortality.

When comparing the subjects with the highest 10th percentile of myocardial performance to the lowest 10th percentile of ejection fraction, myocardial performance index appears to be a superior predictor for cardiovascular mortality (figure 11 and 12). Subjects with both a high myocardial performance index and a low ejection fraction were at especially high risk.

Bella and coworkers previously showed that subjects with an E/A-ratio <0.6 and subjects with an E/A-ratio >1.5 (a restrictive filling pattern) were associated with a 2-fold and 3-fold increase in cardiac mortality, respectively[24]. In this study we did find that subjects with low E/A-ratio had an increased risk of cardiovascular mortality but not the subjects with a restrictive/pseudo-normalized filling pattern. However, as there were only few subjects who were restrictive/pseudo-normalized (n=9), the power to predict mortality in this group was very low.

In conclusion, this study suggests an echocardiographic and Doppler examination to be a valuable tool in determining a patient's risk for cardiovascular mortality, also at the population level.

# Limitations

There are some obvious limitations regarding generalizability in this thesis. As we only examined men of the same age with a similar ethnic background, this study may have limited generalizability to women and other age- and ethnic groups. Furthermore, the cohort may be healthier then the general population as it has been monitored more closely during the follow-up time. However, using a homogenous study population like the ULSAM-cohort eliminates the need to adjust for the possible confounding effects of age, sex and ethnicity and a homogenous population is advantageous when trying to discover associations between genotype and phenotype.

There is always a risk of selection bias when using sub-groups of a cohort, but the different sub-samples in this thesis did not differ in metabolic, anthropometric or hemodynamic variables compared to the rest of the cohort at the age of 70 years and there is no obvious selection bias present.

Other limitations of the study include possible misclassification of cardiovascular mortality, although the accuracy of the Swedish cause-of death-registry has been shown to be high[84].

# Future perspective

## Is modulation of myocardial energy metabolism a future direction for heart failure treatment?

Heart failure treatment of today serves two major purposes, to relieve symptoms and to halt the progression of the disease. Agents like diuretics and digitalis relieve symptoms fast but does not seem to have an effect on the prognosis of the disease over time[85] (figure 13). Agents like ACE inhibitors and beta-blockers have no effect on symptoms in the short term but slow down the progression of the disease and thus lower mortality[6,12] (figure 14). It seems that the decreased mortality after treatment is due to the ability of these agents to lower the neuroendocrine activation seen in heart failure patients[86]. But even though the therapeutic management of heart failure has improved, the mortality and morbidity are still substantial. The reasons why heart failure progresses, even in patients receiving optimal treatment with ACE inhibitors and beta blockers, are not known, but one explanation is that these agents do not sufficiently antagonize all of the biologically active systems that become activated in the setting of heart failure[87].

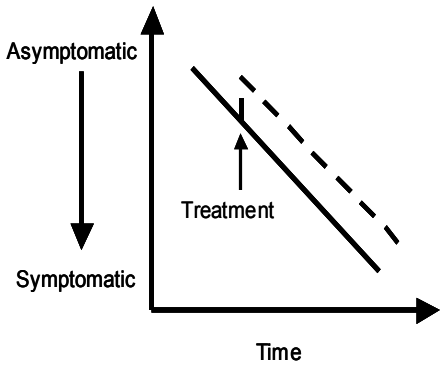


**Figure 13** Treatment with diuretics or digitalis give a fast improvement of symptoms but no effect the long-term prognosis.

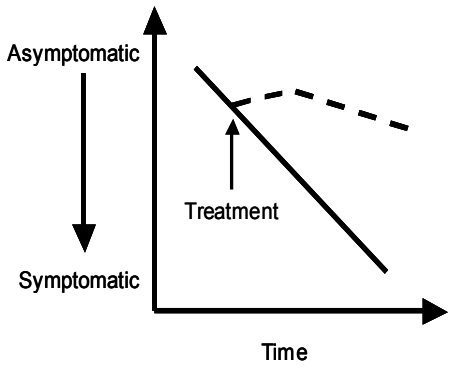


**Figure 14** Treatment with ACE-inhibitors and/or beta-blockers have no short-term benefits but improve symptoms and prognosis in the long term.

Over the last years an accumulating amount of evidence has shown that apart from neuroendocrine activation, heart failure is characterized by metabolic disturbances such as insulin resistance[29] and cachexia[88] and by immune activation with increased expression of cytokines such as tumor necrosis factor (TNF)-alpha[89], interleukin (IL)-1[90] and IL-6[91]. These factors have furthermore been shown to be strong independent predictors of the severity and the prognosis of the disease[31,92]. One plausible direction for future heart failure treatment is to develop new therapeutic strategies that antagonize all the biologically active systems that appear to play a role in the progression of heart failure and by doing so, reduce the morbidity and mortality even further. There are already clinical trials in progress that evaluate the benefit of antagonizing the cytokine activation (TNF-alpha antagonist treatment) and the catabolic/anabolic imbalance (growth hormone treatment) seen in heart failure patients but the results so far have been discouraging[93].

*Thiazolidinediones*

There is a novel group of oral anti-diabetic drugs (thiazolidinediones or glitazones) that bind and activate peroxisome proliferator-activated receptor (PPAR) gamma, a nuclear receptor that regulates the expression of several genes involved in the metabolism. PPAR gamma-activation enhances insulin sensitivity, induce adipogenesis and oppose the expression of cytokines[94], effects that could slow down the progression of heart failure. There are already studies showing improvements of left ventricular function after treatment with thiazolidinediones. Positive inotropic effects have been seen in rat hearts[95] and an increased cardiac output[96] and improved diastolic function[97] have been seen in human diabetic subjects after thiazolidinedione treatment. In non-diabetic pigs, thiazolidinedione treatment improved the recovery of left ventricular systolic and diastolic function after acute ischemia[98]. But the mild fluid retention, manifested as peripheral edema, seen in some subjects treated with thiazolidinediones is a potentially serious side effect in heart failure patients. The mechanism is not clear but it does not seem probable that the increased fluid retention is due to a deleterious effect of thiazolidinediones on the myocardium[99]. The eventual short term worsening of symptoms due to fluid retention caused by peripheral vasodilatation might not affect the prognosis of the disease (see figure 13) and there is reason to believe that possible long-term effects of increased insulin sensitivity, decreased cytokine expression and increased adipogenesis may be beneficial for heart failure patients in the long run. Ongoing trials that address the safety of glitazones treatment in diabetic heart failure patients will answer some of these questions.

*Fatty acid inhibitors*

Improvement on myocardial function has also been seen after treatment with agents that inhibit fatty acid oxidation, i.e. induces an increase in glucose oxidation. In patients with ischaemic heart disease, trimetazidine treatment

has been shown to improve contractile function in several studies[100-102] and short-term treatment with ranolazine improved left ventricular function without an increase in myocardial oxygen consumption, resulting in an increased myocardial mechanical efficiency in dogs with heart failure[103].

*Insulin infusion*

The DIGAMI-trial showed evidence for a beneficial impact on mortality following insulin-glucose infusion after acute myocardial infarction[104] and critically ill patients had a better survival rate and were less likely to require prolonged mechanical ventilation when they had their blood glucose levels normalized with insulin infusions[105]. It is possible that the shift from fatty acid to glucose oxidation after insulin infusion mediated some of the beneficial effect on survival.

These observations provide support for the concept that modulation of energy metabolism in the failing heart may be a useful approach to improve cardiac function and perhaps in the long run, prognosis. Future investigations are necessary to determine whether the alternative approaches, such as thiazolidinediones, fatty acid oxidation inhibitors and glucose-insulin infusion, have a role to play in the future heart failure treatment regimes.

## Future roles for the natriuretic peptides

Since the discovery of ANP, when de Bold et al. infused atrial extracts in rats over twenty years ago[33], almost 12 000 articles have been published regarding the natriuretic peptides. Their role in cardiovascular disease has been extensively examined[106]. Apart from the diuretic, natriuretic and vasorelaxant effects of natriuretic peptides and the involvement in the pathophysiology of hypertension and heart failure, current research have shown an impact of ANP in cardiac and vascular remodeling[107,108]. Moreover, mutations in the ANP gene have been shown to be associated to hypertension and stroke[109,110].

*Natriuretic peptides as markers for left ventricular function*

The usefulness of a screening test is dependent of the accuracy of the test, the prevalence of the disease screened for and the cost of misclassifications (false positives and false negatives)[111]. In recent years, the ventricle-derived hormone brain natriuretic peptide (BNP) and its precursor N-BNP have been shown to be even better markers of left ventricular dysfunction and hypertrophy than ANP and N-ANP[112-114]. Despite that, the use of natriuretic peptides as a screening test for asymptomatic left ventricular dysfunction do not seem warranted in a primary preventive setting where the prevalence of left ventricular dysfunction is low[115]. In contrast, rapid measurement of BNP used in conjunction with other clinical information, seems useful in establishing or

excluding the diagnosis of congestive heart failure in patients with acute dyspnea[116,117]. The natriuretic peptides have also been shown to be relevant as a treatment guide in heart failure patients[118,119].

*The therapeutic potential of the natriuretic peptides*

There was some hope given to a new class of drugs influencing ANP metabolism (vasopeptidase inhibitors) but the results from the clinical trials in heart failure patients and in hypertensive patients did not live up to the expectations[120].

Synthetic B-type natriuretic peptide, or nesiritide, mimics the actions of endogenous BNP and produces a prompt fall in systemic vascular resistance and pulmonary capillary wedge pressure, associated with rapid clinical improvement in decompensated heart failure[121-123]. It is well tolerated; the only major adverse effect known so far is dose-related hypotension[122]. Nesiritide recently gained US Food and Drug Administration approval as parenteral agent for heart failure and may prove to be valuable in the treatment of patients hospitalized for acute decompensated HF.

Instead of simply being a marker for left ventricular function and morphology, the natriuretic peptides have been shown to have therapeutic implications and to play a causal role in the development of cardiovascular disease.

## Possibilities and pitfalls of genetic associations studies

Genetic factors contribute substantially to the development of cardiovascular disease and the publication of the sequence of the human genome[124,125] has enhanced the prospects for identifying genetic variants responsible for disease. Two major strategies have been developed over the last decades to explore genetic determinants of cardiovascular disease, linkage studies and association studies.

Linkage studies examine the co-inheritance of genetic mutations and disease in twins and families. There has been substantial success in this strategy, and several Mendelian syndromes where a single gene mutation causes the disease have been identified[126-128]. But these mutations are rare and often of limited significance to public health.

Association studies examine the relation between different gene alleles and disease/phenotype characteristics in a population. There are several advantages using association studies compared to the traditional linkage analysis. Association studies can be pursued in the absence of knowledge about the inherit-

ance patterns and they do not require a study population with large families where many family members are affected by the disease. Furthermore, association studies have greater statistical power to detect gene effects compared to linkage analysis[129,130] and can detect even moderate effects of gene mutations common in the general population. Despite these advantages, previous association studies have a poor track record for identifying valid associations. An abundance of diverging results of different candidate genes have been published[131]. This may in part be due to inadequate study design, the impact of confounding environmental factors and interactions with other genes and that the impact of single gene mutation on a common disease is often weak in the general population[132].

With millions of SNPs having been identified and new techniques that make DNA determination readily available there are immense possibilities to discover novel genes that influence disease or disease susceptibility. However with this scenario comes concern in how a proper genetic association study should be performed.

The risk of spurious associations in genetic association studies is a major problem. When there are many SNPs reflecting possible variations in a gene and when the prior assumption that a certain allele is non-neutral is weak, the conventionally accepted significance level of $p<0.05$ is likely to represent a false positive. Per definition 1 out of every 20 significance tests will turn out significant just by chance Some authors even suggest that the significance level should be 5 x 10-8 for randomly selected SNPs[130]. Proper power calculations with account taken for the multiple testing are needed and validation in a separate population is essential in order for the results to be convincing. Other ways of reducing the risk of false positive findings is to put emphasis on the biological plausibility of the hypothesis. In other words to choose genes where there already is some knowledge on the function or expression of the gene.

The most important potential confounder in genetic epidemiology is probably ethnicity and a careful selection of cases and controls from a homogenous population is crucial in order to avoid selection bias.

There is a high risk of false negative results as there are so many SNPs in one gene that it is easy to miss the functional polymorphism. That no relation was found between disease and a specific SNP does not necessarily mean that the gene itself is not involved in the disease process. To small samples have in some cases been the causes of false negative findings in the past. Large samples are needed in order to detect even modest genetic contributions.

There are several approaches for SNP genotyping and the method used should be robust, accurate and involve a verification procedure where an error rate is stated. The choice of phenotype is also very important. Without a well-defined, reliable, and clinically and physiologically relevant phenotype an even-

tual association will not give proper insight to biological plausibility. Furthermore, determination of appropriate phenotypic confounders may improve the validity of the study. No genetic association study is better then the phenotype characteristics used.

Of course, all studies cannot meet all of these criteria but the interpretation of the result should be balanced according to these suggestions. Ultimately, genetic association studies together with functional studies in vitro and in animals will determine the plausibility that the mutation in the candidate gene is causal and affects the disease process.

**Considerations for genetic association studies**

| **Study design** | **Study Population** |
|---|---|
| Biologically plausible hypothesis | Large samples |
| Proper power calculations | Ethnical homogeneity |
| Account taken for multiple testing | |
| Adjustment for possible confounders | |
| Validation in an independent population | |
| **Methods** | **Report** |
| High quality genotyping | Balanced interpretation |
| Well defined phenotype characteristics | |

The future direction for cardiovascular genetic research is a polygenic approach where gene-gene interaction is explored. The above considerations will be even more important when combinations of several genes are involved, as there is greater complexity in the biological rationale for which genes or which SNPs to combine.

## What does the Myocardial performance index really reflect?

The echocardiographic and Doppler measurements of cardiac function reflect different aspects of the cardiac cycle and have different strengths and limitations in terms of validity and reproducibility. As the myocardial performance index is a fairly new measurement of cardiac function few studies have addressed what the Doppler index really reflects.

*Myocardial aspects of Myocardial performance index*

The isovolumic contraction time corresponds to when calcium enters the myoplasm from the sarcolemma, while the isovolumic relaxation time reflects the removal of $Ca^{2+}$ from the myoplasm by $Ca^{2+}$-ATPases. Thus the myocardial performance index mirrors both the depolarization and repolarization of the myocardial cells. It seems like changes in cellular $Ca^{2+}$ handling in the myocardium underlie much of the abnormal contractility and relaxation[133]. In the failing heart, the contraction and relaxation becomes slower[134] which explains why the myocardial performance indexes increases with deterioration of cardiac function. This is supported by a recent study where dobutamine administration to heart failure patients improved the myocardial performance index, by decreasing the isovolumic relaxation and contraction times[135].

In a previous study comparing the myocardial performance index to simultaneous cardiac catheterization measurements of left ventricular function, myocardial performance index was found to reflect both systolic and diastolic function[17]. The ratio of isovolumic contraction time and ejection time was closely correlated to +dP/dt (reflecting systolic function) and the ratio of isovolumic relaxation time and ejection time was closely correlated to-dP/dt and tau (reflecting diastolic function). In other words, the myocardial performance index could be considered the sum of an index reflecting systolic function and an index reflecting diastolic function.

The myocardial performance index is also negatively correlated to oxygen uptake at peak exercise and at the anaerobic threshold, and a predictor of cardiopulmonary exercise capacity independent of other echocardiographic measurements[135]. Consequently, in clinical practice the myocardial performance index has been shown to be a sensitive indicator for symptomatic heart failure[136].

An advantage of the Doppler index is that it is fairly independent of heart rate. Patients with sick sinus syndrome treated with a pacemaker were paced at increasing rates from 50 to 100 beats per minute. The myocardial performance index increased on average 0.02 per 10 beats per minute increase[137]. In the ULSAM cohort there was a weak positive correlation between heart rate that did not quite reach statistical significance (unpublished data, Ärnlöv et al.). Thus, heart rate does not seem to affect the myocardial performance index in a clinically significant manner.

*Peripheral aspects of the Myocardial Performance Index*

Apart from being a question of direct myocardial performance, the function of the heart is dependent of loading conditions. Little is known whether preload and afterload affects myocardial performance index.

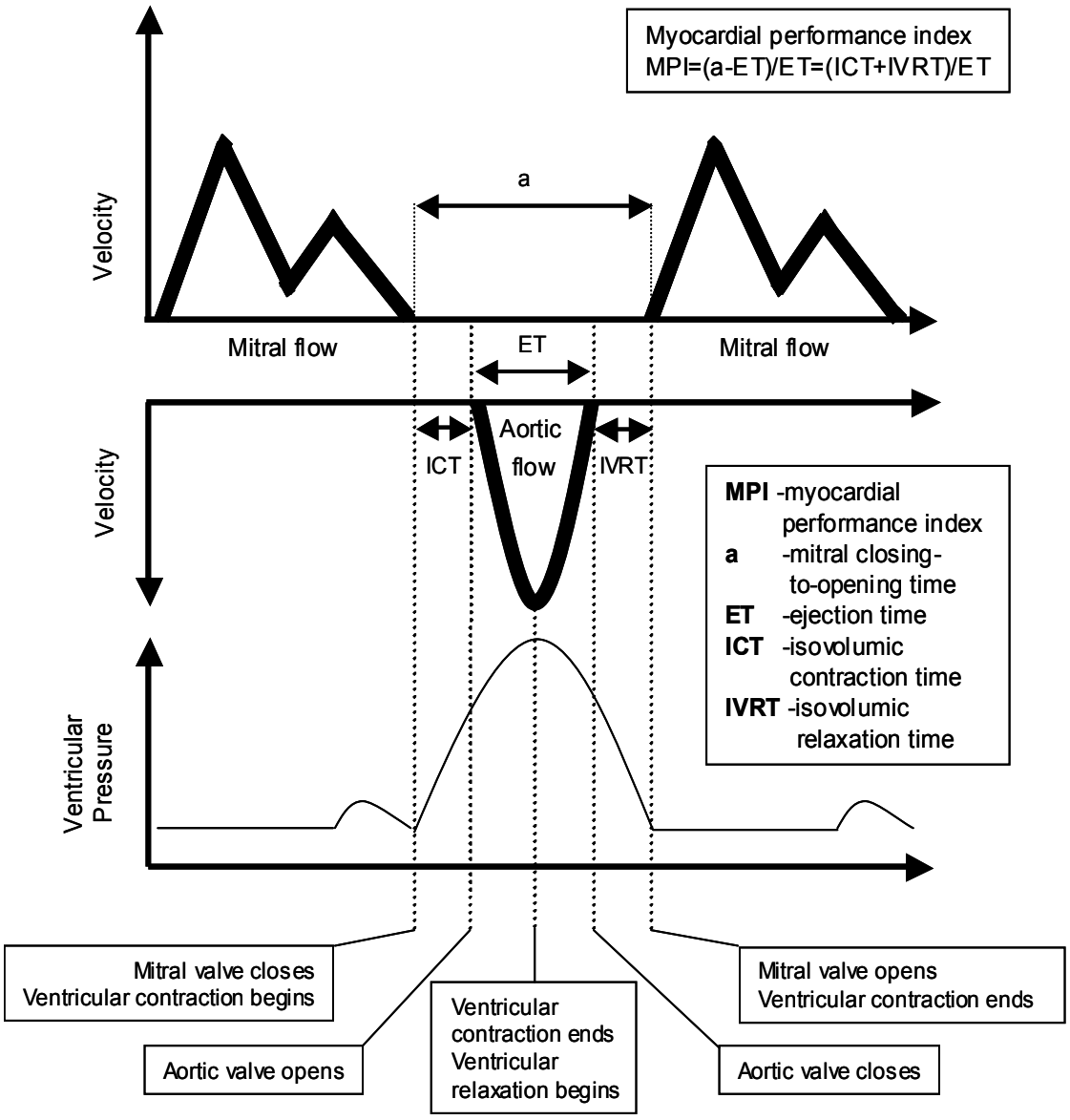


**Figure 15** The relation between left ventricular pressure and the myocardial performance index

*Preload*

In a study by Møller and coworkers, statistically but not clinically significant effects on Myocardial performance index after preload alterations in healthy subject were seen but no effect of preload alterations in subjects with a myocardial infarction[138].

*Afterload*

No studies have been performed on the effects of afterload on myocardial performance index but it seems reasonable that there may be some influence of peripheral resistance.

Doppler echocardiography does not measure the actual contraction or relaxation time; it measures the interval between the closure of the mitral valve and opening of the aortic valve and vice versa (i.e. the isovolumic relaxation and contraction, see figure 15) and can thus only be considered a mirror of the actual myocardial contraction and relaxation time. The actual myocardial contraction continues on after the aortic valve opens and the actual myocardial relaxation begins before the aortic valve closes. With an increased afterload, a higher pressure is needed in order to open the aortic valve. The ventricle takes longer time to reach sufficient pressure, resulting in a prolonged isovolumic contraction time and a shortened ejection time as measured by Doppler. Consequently, due to the increased afterload the aortic valve closes sooner which shortens the ejection time further and prolongs the measured isovolumic relaxation time. Thus alterations in afterload may affect the echocardiographic reflection of the contraction and relaxation intervals even though it might not affect the actual contraction and relaxation times. Inotropic mechanisms (such as Frank-Starling and sympathetic drive) probably compensates for some of this effect in healthy individuals but not in patients with left ventricular dysfunction where no extra inotropic power is given by these compensatory mechanisms.

The hypothesis is supported by the strong positive correlation between myocardial performance index and the total peripheral resistance index and mean arterial pressure and the negative correlation between myocardial performance index and stroke volume/pulse pressure-ratio (reflecting arterial compliance) found in the ULSAM cohort (unpublished data, Ärnlöv). However, in order to properly address the issue of whether afterload affects the myocardial performance index or not, invasive studies are needed.

*Why did not the traditional risk factors predict cardiovascular mortality when account was taken for the Myocardial Performance Index?*

In paper IV none of the traditional risk factors (previous myocardial infarction, left ventricular hypertrophy, hypertension, hyperlipidemia, diabetes, and smoking) remained significant predictors of cardiovascular mortality in a multivariate Cox proportional hazard ratio model that included the myocardial performance index. One explanation may be that the pathological changes induced by these risk factors in the myocardium and in the peripheral vessels are reflected by the Doppler index.

Large artery stiffness has recently been related to cardiovascular morbidity and mortality[139,140] and it is apparent that stiffening of central arteries may lead to increased afterload. As discussed above there may be an influence of afterload on the myocardial performance index. Thus, the increased afterload present in hypertension, hyperlipidemia, diabetes, left ventricular hypertrophy and coronary heart disease[141,142] may affect the Doppler index and consequently reflect the increased risk of mortality due to the vascular pathology caused by these diseases.

There may also be direct effects of these risk factors on myocardial function independent of coronary atherosclerosis. Left ventricular hypertrophy, hypertension and diabetes are all associated with a prolonged IVRT[143-146] and diabetics showed a shortened left ventricular ejection time, longer pre-ejection period, and a higher ratio of pre-ejection period/left ventricular ejection time[147]. These effects are likely to influence the myocardial performance index.

In conclusion, apart from being an easily assessable, reliable indicator of global cardiac function, the myocardial performance index may be a marker for the vascular and myocardial pathology caused by cardiovascular risk factors.

# Conclusions

Several factors associated with the insulin resistance syndrome predicted left ventricular systolic dysfunction independent of myocardial infarction, hypertension, diabetes and the use of cardiovascular medication after twenty years follow-up.

Plasma N-ANP levels were significantly increased in subjects with left ventricular dysfunction compared to healthy subjects. However, the diagnostic accuracy was poor due to the extensive overlapping between the groups.

SNPs and haplotypes of the novel hUNC-93B1 gene were discovered and relations between the SNP vp94 and haplotype H3 of the hUNC-93B1 gene and the E/A-ratio were found and validated.

Myocardial performance index (a Doppler derived index of combined left ventricular systolic and diastolic function) and left ventricular ejection fraction were found to be predictors for cardiovascular mortality independent of traditional cardiovascular risk factors in a longitudinal analysis with a mean follow-up of seven years.

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