Emerging biomarkers in cardiometabolic disease

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Abstract

The epidemiological association between diabetes and heart failure is well-established and the two entities are emerging as global threats, both individually and synergistically, to an aging population. The exploration of multiple proteins can shed light on pathophysiological pathways in both diabetes and cardiovascular disease. This can possibly provide novel diagnostic, prognostic and hopefully therapeutic implications.

The overall aim of this dissertation was to explore novel biomarkers in cardiometabolic disease especially in heart failure.

This thesis was based on epidemiological data from the Malmö Preventive Project Re-Examination (MPP-RES) study and the Heart and brain failure investigation trial (HARVEST-Malmö). In **Paper 1** we used a multiplex proteomic panel consisting of 92 proteins with known or alleged associations with cardiovascular disease, metabolism and inflammation to explore novel biomarkers for incident diabetes in the population-based cohort MPP-RES (n=1026). We were able to identify seven proteins associated with incident diabetes, of which four not previously described. Two of these (Galectin-4 and Paraoxonase-3) were associated with diabetes independently of fasting plasma glucose implying an glucose-independent association with diabetes.

In **Paper 2**, we built upon the results from **Paper 1**, since one of our ultimate aims with this thesis was to explore common pathophysiological pathways between diabetes and cardiovascular disease. Using the seven proteins identified in **Paper 1** we investigated whether these were associated with pertinent cardiovascular outcomes such as all-cause and cardiovascular mortality, incident coronary events and incident heart failure. We found that two proteins (Galectin-4 and Cathepsin D) were associated with all investigated outcomes in multivariable Cox regression analyses and represented novel findings. Galectin-4 may possibly exert its effect on cardiometabolic disease through the incretin system and Cathepsin-D has previously been described to reduce the antioxidative effects of high-density lipoprotein.

In **Paper 3**, we switched from the population-based MPP-RES cohort to the HARVEST-Malmö study which consists of heart failure patients admitted to the cardiology and internal medicine wards at Skånes University Hospital in Malmö, Sweden. We assessed the predictive ability in terms of mortality and re-hospitalization, of five different proteins (midregional pro-adrenomedullin, copeptin, NT-proBNP, CT-pro-endothelin-1 and cystatin C). The investigated proteins represent different pathophysiological mechanisms involved in heart failure such as the neuroendocrine response, cardiovascular stress and renal function. Higher plasma levels of all proteins but CT-pro-endothelin-1 were associated with increased risk of post-discharge mortality but only NT-proBNP, which in many ways is the gold standard for biomarkers in heart failure, was associated with increased risk of re-hospitalization.

Finally, in **Paper 4,** which was a collaboration with an Italian heart failure study (GREAT Rome Network) we investigated the effects of two emerging biomarkers in heart failure; bioactive adrenomedullin (bio-ADM) which is considered a marker for congestion, and proenkephalin A (penKid), which is a marker for renal dysfunction. While NT-proBNP has many uses, it has not been shown to adequately assess residual congestion in heart failure patients. We were able to show that increased levels of bio-ADM were associated with increased congestion measured through a clinical congestion score where peripheral edema was the strongest and driving association. Furthermore, we showed that bio-ADM was predictive of 1-year mortality, increased risk of re-hospitalization and length of hospital stay. PenKid levels responded approximately 48 hours prior to creatinine in the setting of acute kidney injury and we showed that penKid was associated with worsening renal function as well as with in-hospital and 1-year mortality.

Key words: biomarkers, cardiometabolic disease, diabetes, heart failure, proteomics

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List of papers

This thesis is based on the following four original papers.

1. Molvin J, Pareek M, Jujic A, et al. Using a Targeted Proteomics Chip to Explore Pathophysiological Pathways for Incident Diabetes- The Malmö Preventive Project. Sci Rep. 2019;9(1):272. Published 2019 Jan 22. doi:10.1038/s41598-018-36512-y
2. Molvin J, Jujić A, Melander O, Pareek M, Råstam L, Lindblad U, Daka B, Leósdóttir M, Nilsson PM, Olsen MH, Magnusson M. Proteomic exploration of common pathophysiological pathways in diabetes andcardiovascular disease ESC Heart Fail. 2020 Oct 13. doi: 10.1002/ehf2.13036. Online ahead of print.
3. Molvin J, Jujic A, Bachus E, et al. Cardiovascular biomarkers predict post-discharge re-hospitalization risk and mortality among Swedish heart failure patients. ESC Heart Fail. 2019;6(5):992-999. doi:10.1002/ehf2.12486
4. Molvin J, Jujic A, Navarin S, et al. Bioactive adrenomedullin, proenkephalin A and clinical outcomes in an acute heart failure setting. Open Heart. 2019;6(2):e001048. Published 2019 Jul 3. doi:10.1136/openhrt-2019-001048

Scientific contributions that are not included in this thesis are as follows:

- Holm H, Bachus E, Jujic A, Nilsson ED, Wadström B, Molvin J, Minthon L, Fedorowski A, Nägga K, Magnusson M. Cognitive test results are associated with mortality and rehospitalization in heart failure: Swedish prospective cohort study. ESC Heart Fail. 2020 Aug 18;7(5):2948-55. doi: 10.1002/ehf2.12909. Online ahead of print.
- Molvin J, Jujic A, Melander O, Pareek M, Råstam L, Lindblad U, Daka B, Leosdottir M, Nilsson P, Olsen M, Magnusson M. Exploration of pathophysiological pathways for incident atrial fibrillation using a multiplex proteomic chip. Open Heart. 2020 Mar 15;7(1):e001190. doi: 10.1136/openhrt-2019-001190. eCollection 2020.
- Molvin J, Jujic A, Nilsson PM, Leosdottir M, Lindblad U, Daka B, Bennet L, Råstam L, Lyssenko V, Magnusson M. A diabetes-associated genetic variant is associated with diastolic dysfunction and cardiovascular disease. ESC Heart Fail. 2020 Feb;7(1):348-356. doi: 10.1002/ehf2.12573. Epub 2019 Dec 20.
- Ali A, Holm H, Molvin J, Bachus E, Tasevska-Dinevska G, Fedorowski A, Jujic A, Magnusson M. Autonomic dysfunction is associated with cardiac remodelling in heart failure patients. ESC Heart Fail. 2018 Feb;5(1):46-52. doi: 10.1002/ehf2.12223. Epub 2017 Sep 28.
- Christensson A, Grubb A, Molvin J, Holm H, Gransbo K, Tasevska-Dinevska G, Bachus E, Jujic A, Magnusson M. The shrunken pore syndrome is associated with declined right ventricular systolic function in a heart failure population - the HARVEST study. Scand J Clin Lab Invest. 2016 Nov;76(7):568-574. doi: 10.1080/00365513.2016.1223338.
- Magnusson M, Molvin J, Engström G, Svensson-Färbom P, Persson M, Christensson A, Nilsson P, Melander O. Cystatin C and Risk of Diabetes and the Metabolic Syndrome - Biomarker and Genotype Association Analyses.PLoSOne.2016May24;11(5):e0155735.doi:10.1371/journal.pone.0155735. eCollection 2016

Abbreviations

ACEi	Angiotensin converting enzyme inhibitor
ACM	All-cause mortality
ACS	Acute coronary syndrome
ADM	Adrenomedullin
AHT	Anti-hypertensive treatment
ARB	Angiotensin receptor blocker
Bio-ADM	Biologically active adrenomedullin
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CatD	Cathepsin D
CD163	Scavenger receptor cysteine rich type 1 protein M130
CE	Coronary events
CMD	Cardiometabolic disease
CT-pro-ET-1	C-terminal pro-endothelin 1
CVD	Cardiovascular disease
CVM	Cardiovascular mortality
CXR	Chest X-ray
DBP	Diastolic blood pressure
DM	Type 2 Diabetes Mellitus
DPP4	Dipeptidyl peptidase-4
ET-1	Endothelin-1
FABP4	Fatty acid binding protein-4
FFA	Free fatty acids

FPG	Fasting plasma glucose
FUT	Follow-up time
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GREAT	Global research on acute conditions team
HARVEST	Heart and brain failure investigation trial
HbA1c	Glycosylated haemoglobin A1c
HDL	High density lipoprotein cholesterol
HF	Heart failure
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IGFBP-2	Insulin-like growth factor binding protein-2
IR	Insulin resistance
MPP	Malmö Preventive Project
MPP-RES	MPP-Re-examination study
MR-proADM	Midregional pro-adrenomedullin
NP	Natriuretic peptides
NT-proBNP	N-terminal pro-B type natriuretic peptide
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PEA	Proximity extension assay
PG	Plasma Glucose
PON3	Paraoxonase 3
SGLT2	Sodium glucose co-transporter 2
SBP	Systolic blood pressure
TG	Triglyceride
TTE	Transthoracic echocardiography
WRF	Worsening renal function

Introduction

Historical context of cardiometabolic disease

More than 3000 years ago, the ancient Egyptians noted that black ants were attracted to the urine of patients with excessive thirst and diuresis. They also noted that these patients didn't fare too well (1). They called the condition *madhumeha* or "honey urine". The English doctor Thomas Willis (1621-1675) was later credited for coining the term *diabetes mellitus* from the Greek word *diabetes* meaning "to pass through" and adding the Latin word *mellitus* meaning "honey" after sampling or rather oversampling the urine of diabetic patients, and famously stating that it was "*wonderfully sweet as if it were imbued with honey or sugar"* (1).

In 1881, Ernst von Leyden, a German pathologist and director of the famous Charité hospital in Berlin, remarked that *"heart failure is a frequent and noteworthy complication of diabetes mellitus"* (2) and this observation was reiterated and extended by the Danish internist Knud Lundbaek in 1954 when he described the advanced atherosclerosis and subsequent end-organ damage in diabetic subjects as a vascular disease or *diabetic angiopathy* (3).

The plot thickened however, when Rubler in a seminal study from 1972 comprising of only four patients demonstrated diabetes-related nephropathy and cardiomyopathy in the absence of any valvular, congenital or hypertensive heart disease, alcoholism or most importantly coronary artery disease (CAD), introducing the term *diabetic cardiomyopathy* (4).

The Framingham Heart study started in 1948 as a response to the staggering mortality of cardiovascular disease (CVD) accounting for one in two deaths in the US (5). The results, showing a threefold cardiovascular mortality rate in subjects with diabetes (6) and a fivefold risk of incident heart failure (HF) in diabetic women(7) independently of known risk factors, prompted Jarrett in 1984 to ask the question if diabetes was the chicken or the egg (or neither) in CVD (8), suggesting instead that diabetes and CVD share common precursors.

This idea together with the similar *Syndrome X* introduced by Reaven (9) was united by Stern in 1995 who introduced the *common soil hypothesis* (10) which proposes insulin resistance (IR) as the driving force behind hypertension, obesity, dyslipidemia and ultimately CVD.

Cardiometabolic disease

The constellation of disturbed glucose metabolism, hypertension, dyslipidemia and abdominal obesity resulting in CVD constitutes *cardiometabolic disease* (CMD) although there are some variations in the exact definition (11). In this thesis I refer to CMD in the more general sense i.e. the connection between dysglycemia (with an emphasis on diabetes mellitus), CVD and mortality.

The WHO defines CVD as the collective term for a group of disorders affecting the heart and blood vessels including hypertension, CAD, stroke, peripheral vascular disease, HF, rheumatic heart disease, congenital heart disease and various cardiomyopathies (12). However, in this thesis, CVD references CAD manifesting in coronary events (CE), heart failure (HF) and cardiovascular mortality (CVM).

Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disease defined as a condition of chronic hyperglycemia. There are two main types of DM; type 1 DM which is characterized by the autoimmune destruction of insulin-producing beta-cells in the pancreas and type 2 DM which is typically the effect of insulin resistance and a relative deficiency of insulin. This form of DM constitutes more than 90 percent of diabetes cases worldwide (13).

This thesis will focus solely on type 2 DM and will be further referenced only as DM.

Diagnosis

DM is most often diagnosed based on plasma glucose (PG) criteria; either a fasting PG (FPG) value of $\geq$7.0 mmol/l on at least two different occasions, a PG > 11.1 mmol/l two hours after an oral glucose tolerance test (OGTT) or a random PG of >11.1mmol/l(14).
Glycosylated haemoglobin A1c (HbA1c) which is the foremost method of glycemic management in diabetic subjects (15) can also be used to diagnose DM (14). DM is preceded by a period of abnormal glucose homeostasis classified as either impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) that commonly represent degrees of IR.

European and American guidelines recommend screening for DM in subjects over 45 years of age or younger if risk factors such as obesity, family history of DM or hypertension are present(14, 15).

Epidemiology

The global prevalence of DM has risen from approximately 100 million in 1980 to more than 400 million in 2014 (16). By the latest estimates (2019) half a billion people between the ages of 20 and 79 years are currently living with DM, representing 9.3 percent of the population in this age group(17). The projections for the next decades are even more dismal with the global prevalence predicted to rise to 578 million (10.2%) by 2030 and to 700 million (10.9%) by 2045. The largest increases are predicted to occur in countries moving from low-income to middle-income status with China and India as the main epicentres (17, 18). The prevalence is slightly lower in women but like in men, is also projected to increase over the next few decades (17, 19).

In 2019 an estimated 4.2 million deaths, one in nine deaths, in the age group 20-79 years were attributable to DM and almost half occurred in people below the age of 60 years (20) which underlines the necessity of early identification, prevention and intervention of high-risk individuals.

An aging, increasingly obese population leading a sedentary lifestyle and consuming an energy-dense diet is the main explanation for the diabetes epidemic although genetic predisposition is also an important factor (14, 21). However, aggressive lifestyle interventions have been shown to reduce the incidence of DM in high-risk individuals (22).

Diabetic complications

Complications arising from DM can chiefly be divided into microvascular and macrovascular complications. The microvascular complications (i.e. retinopathy, nephropathy and neuropathy) are closely related to the level and the duration of hyperglycemia (23) and there is a clear benefit of intensive glucose management (24, 25). However, the relationship between intensive glucose management and reduced macrovascular complications (i.e. CVD) is not equally clear although new treatment options in the form of glucagon-like peptide 1 agonists (GLP-1) and sodium glucose co-transporter 2 inhibitors (SGLT-2) offer promise (26-30).

For the purposes of this thesis I will focus solely on the cardiac macrovascular complications of DM which are mainly coronary events and heart failure.

Cardiovascular disease

CVD remains the number one global killer with almost 18 million people dying from CVD in 2016 representing 31% of all global deaths. Of these deaths, 85% are due to myocardial infarction and stroke (31).

When looking at CVD in diabetic subjects the numbers get even bleaker. In subjects with DM, CVD occurs more frequently and earlier in life and a diagnosis

of DM is the risk equivalent of aging 15 years (32). DM confers up to a quadrupled risk of cardiovascular mortality and morbidity (13, 33). Furthermore, diabetic arteries are prone to a more general and accelerated atherosclerotic process, which results in a narrower lumen, a higher plaque burden, and more vulnerable plaques that are more likely to rupture and lead to coronary events (34). In addition to this, hypertension is almost three times more common in diabetic subjects further contributing to the increased risk of CVD in DM (35).

For HF the numbers are even more discouraging. In men, DM doubles the risk of developing HF and in women the risk has been suggested to be fivefold (7, 36, 37). Furthermore, the prognosis in subjects with concomitant DM and HF is significantly worse than in subjects with HF alone (38).

In general, the risk for coronary artery disease is lower in women compared to men but this difference disappears in the presence of DM (39).

The increased incidence of HF in patients with DM is present even after adjusting for risk factors such as age, CAD and hypertension and is, as mentioned previously, sometimes referred to as diabetic cardiomyopathy (4).

Subjects with chronic HF often show IR, even in the absence of DM, as a sign of abnormal energy metabolism. This HF-induced IR, not only increases risk of DM, but has also been shown to be an independent predictor of mortality and it has been suggested that targeting IR can be beneficial for patients with HF (40). This creates a bidirectional relationship between DM and HF with each disease increasing the risk of the other (41).

This relationship could partly be explained by the metabolic alterations seen in both DM and HF. In IR and DM there is an increased release of free fatty acids (FFA) due to increased lipolysis which leads to a decrease of myocardial glucose uptake (42). This change in substrate availability leads to excessive cardiac FFA-oxidation exceeding the cardiac capacity leading to triglyceride (TG) accumulation within the cardiomyocytes, production of toxic lipid intermediates and ultimately higher oxygen consumption and reduced cardiac efficiency(43).

In chronic HF, in the absence of IR or DM, there is an initial shift to preferred glucose metabolism over FFA which initially improves myocardial contractile efficiency (44). However, as HF progresses there is another shift to predominantly FFA metabolism which resembles that seen in subjects with IR or DM as described above with the same dire outcomes (45).

Furthermore, the diabetic cardiomyocytes have a disturbed intracellular calcium metabolism leading to increased cytosolic calcium concentrations resulting in impaired relaxation (46). This ultimately leads to diastolic dysfunction which is considered the earliest manifestation of diabetes-induced left ventricular dysfunction (47).

As described above, from an epidemiological perspective the cardiovascular complications of DM are well-established but the underlying pathophysiological mechanisms are complex and still not fully understood (48).

This global cardiometabolic tsunami of increasing IR and DM and resulting premature death and disability calls for better preventive and therapeutic measures, especially identifying high-risk individuals given an estimated 50% of diabetic individuals are undiagnosed (17).

Definition of a biomarker

The World Health Organization states that "A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (49).

This broad definition highlights the fact that the term and use of biomarkers can span from something as simple as measuring the heart rate or body temperature to complex laboratory tests or modern imaging methods.

Biomarkers can furthermore be classified into diagnostic, prognostic or predictive. For instance, in cardiology we use troponin for the diagnosis of myocardial infarction (50) and natriuretic peptides for staging and ruling out congestive heart failure (51). A prognostic biomarker will provide information about the likely outcome of a disease in an individual regardless of treatment, whereas a predictive biomarker can help identify individuals who are most likely to respond to a specific treatment (52). The use of predictive biomarkers is an appealing approach to individualized or precision medicine which by many is considered the future of biomarker research (53) and was highlighted by President Obama in his State of the Union address in 2015.

> Tonight, I'm launching a new Precision Medicine Initiative to bring us closer to curing diseases like cancer and diabetes — and to give all of us access to the personalized information we need to keep ourselves and our families healthier.
>
> — President Barack Obama, State of the Union Address, January 20, 2015

Another application of biomarkers is to use them as surrogate endpoints in clinical trials in lieu of *true* or *hard* endpoints such as mortality or incident heart failure. This use has the advantage of reducing sample size and allowing for shorter follow-up periods (54). However, there are pitfalls to this practice. An example was the use of high-density lipoprotein cholesterol (HDL) as a surrogate endpoint instead of true cardiovascular outcomes. We know from epidemiological studies that an increase of HDL is associated with lower risk of CAD (55) but numerous clinical studies

with different HDL-raising pharmacological therapies have been ineffective in reducing cardiovascular events and in some cases even hazardous to the patients (56).

It is important to remain humble when interpreting results and drawing conclusions from biomarker studies including the results from this thesis, as biomarkers could really only serve as replacements for clinical outcomes if we fully understand the underlying pathophysiology which we rarely, if ever, truly do. This need for humility was expressed by the physician and philosopher Isaac Judaeus more than a 1000 years ago when commenting on the practice of uroscopy, the visual interpretation of urine (57).

> But in our time there are fools who would base prophecies on it, without seeing the patient, and determine what disease is present and whether the patient will die and other foolishness.

Role of predictive biomarkers

The complexity and diversity of pathophysiological mechanisms of CMD combined with the advent of advanced large scale "–omic" studies has resulted in an abundance of potential biomarkers which is illustrated in **Figure 1** (58).

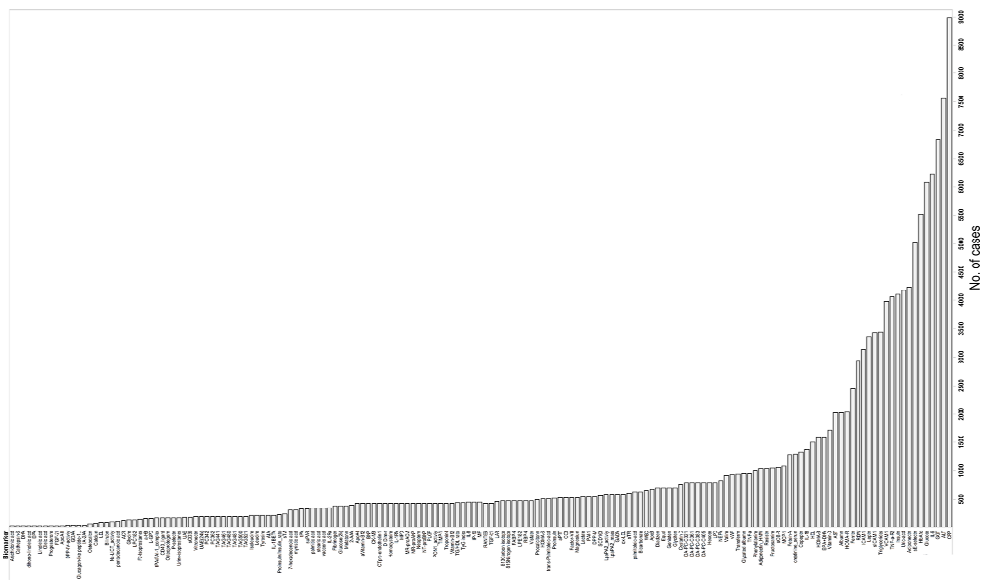

Figure 1. Identified Biomarkers for the Risk of Developing DM. The height of bars denotes total number of incident DM cases across all studies for each biomarker.(58)

However, with the exception of plasma glucose (PG) and HbA1c, which are strong DM predictors as a result of being part of the diagnosis, current biomarkers routinely used in clinical practice (e.g. obesity) are insufficiently precise predictors of DM.

Omics is a collective term for various disciplines in biology such as genomics, proteomics, lipidomics, transcriptomics and metabolomics all of which can provide insight into pathophysiological mechanisms and also identify novel biomarkers.

Protein profiling

Affinity-based immunoassays which uses antibodies linked to various reporters (e.g. fluorescence, radioactivity, enzymatic activity) remain the gold standard for the quantification and identification of proteins but have limitations regarding low-abundance proteins, cross-reactivity and high cost (59). A relatively recent development in protein profiling is called proximity extension assay (PEA). This method uses oligonucleotide acids to label two antibodies and then polymerase chain reaction (PCR) to amplify, detect and quantify (semi-quantitatively) proteins which allows for high specificity and sensitivity for low-abundance proteins in small sample volumes (60). This process is illustrated in **Figure 2** (60).

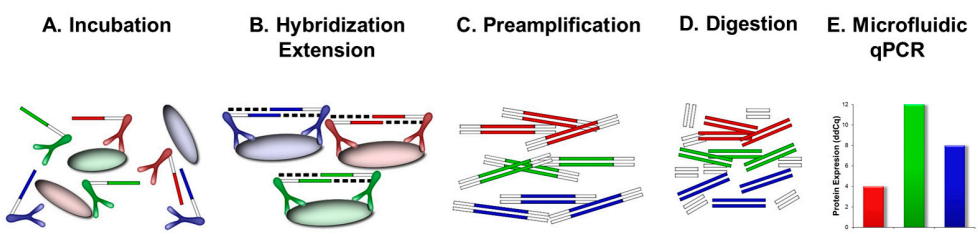

Figure 2. (A) Specific antibodies are equipped with oligonucletotides (PEA probes) and mixed with an antigen-containing sample. **(B)** All PEA probes bind their specific antigens and the oligonucleotides hybridize. Addition of a DNA polymerase leads to an extension and formation of a PCR template. (**C**) Universal primers are added. (**D**) An enzyme digests the DNA templates and remove all unbound primers. (**E**) Finally each individual DNA sequence is detected and quantified by microfluidic qPCR. Picture and descirption adapted from (60).

The PEA method has recently been compared to conventional immunoassays with excellent correlations both for biomarker levels as well as clinical outcomes making PEA a fitting choice for biomarker screening (61).

In **paper I** and **II** we used such a targeted multiplex protein platform called the Olink ProSeek Multiplex CVD III panel which consists of 92 proteins selected by leading experts in the field, with either established or proposed association with CVD, inflammation and metabolism (62-64).

In **paper III** and **paper IV** we investigated the prognostic role of both established and emerging biomarkers in an acute HF setting. Biomarkers reflecting different aspects of HF such as, cardiovascular stress, neurohormonal activation and renal function, were studied. Their pathophysiological mechanisms and possible connections to CMD are examined in the *Discussion* section of this thesis.

Aims

Overall aims

To explore diagnostic and prognostic biomarkers in cardiometabolic disease in population-based and heart failure cohorts.

Specific aims

Paper I: To explore potential biomarkers for incident diabetes in a population-based cohort using a multiplex proteomic panel

Paper II: To explore how our findings from **Paper I** associate with cardiovascular disease and mortality in order to identify novel biomarkers in cardiometabolic disease

Paper III: To assess the prognostic role of biomarkers of cardiovascular stress, neuroendocrine response and renal function in an acute heart failure population

Paper IV: To examine the diagnostic value of bio-ADM in congestion and penKid in worsening renal function and their prognostic value regarding mortality, re-hospitalization and length of hospital stay in two separate European acute heart failure cohorts

Materials and methods

Study populations

Malmö Preventive Project, MPP

During 1974 -1992, birth cohorts, between 1921 and 1949, of inhabitants in Malmö, Sweden, were invited to participate in a large cohort study, i.e., the Malmö Preventive Project (MPP), with a total of 33,346 individuals attending in order to explore cardiovascular risk factors, alcohol consumption, glucometabolic disturbances and breast cancer (65). At baseline, participants underwent clinical examination regarding hypertension, DM, obesity, dyslipidemia, smoking and family history.

Re-examination of 18,240 surviving MPP participants, the MPP Re-Examination Study (MPP-RES), was conducted during 2002-2006 (63% men, 72% attendance rate, mean age 69±6 years).

In a subsample of the MPP-RES (1,792 participants), echocardiography and ECGs were recorded, further referenced as *MPP-RES echo*. These subjects were randomly selected from groups defined by glucometabolic status: normal fasting glucose; impaired fasting glucose; new onset DM; and prevalent DM, with oversampling from the groups with glucometabolic disturbances to ensure numerical balance between the groups (66).

All individuals participating in MPP and MPP-RES gave written informed consent and ethical approval was given by the Regional Ethics Board in Lund, Sweden.

The heart and brain failure investigation study, HARVEST-Malmö

HARVEST-Malmö started in March 2014 and is an ongoing study undertaken in patients hospitalized for HF (ICD-10: I50-) in Skåne University Hospital, Malmö (67). The inclusion criteria are admission to the Department of Cardiology or Internal Medicine for treatment of newly diagnosed or exacerbated HF regardless of etiology, duration or severity. The aim of the study is to identify markers and mechanisms for progressive HF and associated cognitive dysfunction in order to improve treatment, prognostication and potentially identify novel therapeutic targets.

Subjects undergo clinical examination and blood samples are drawn after overnight fast. Subjects also undergo a number of cognitive tests and all subjects are examined with transthoracic echocardiogram to assess cardiac function. Eligible subjects are invited to a follow-up examination and echocardiogram six months after hospital discharge. As of 2020, almost 500 subjects have been included in HARVEST-Malmö.

The only exclusion criterion is the inability to give informed consent. In the case of severe cognitive impairment, informed consent is collected from relatives. The study has been approved by the ethical review board at Lund University, Sweden and complies with the Declaration of Helsinki.

GREAT Network Rome (Global Research on Acute conditions team)

The GREAT Association is an International Network between experts operating in the management of acute clinical conditions (68). Between May 2013 and March 2015, 245 patients that were referred to the emergency department of Sant'Andrea hospital in Rome, Italy for HF symptoms and signs and who received a final diagnosis of new onset or worsening HF were enrolled to the GREAT Network Acute Heart Failure Rome Study. Inability to consent to the study was the only exclusion criteria. The study protocol complied with the Declaration of Helsinki and a written informed consent was obtained from all participants.

Paper-specific methods

Paper I

In **paper I** we investigated whether proteins from Olink ProSeek CVD III multiplex proteomic panel predict incident DM in MPP-RES echo (n=1792). Plasma samples from 1737 individuals were successfully analyzed. Patients with missing covariates at baseline (n = 30) and prevalent DM (n = 681) were excluded, resulting in 1026 eligible subjects for the main analyses of incident DM. 146 subjects developed DM during the median follow-up time (FUT) of 8.0 years. A flowchart of the study population of **paper I** and **II** is provided in **Figure 3.**

Paper II

In this paper we investigated how the seven proteins associated with incident DM identified in **paper I** are associated with incident HF, incident coronary events (CE), cardiovascular mortality (CVM) and all-cause mortality (ACM) in the MPP-RES echo cohort. Plasma samples from 1737 individuals were successfully analysed. For analyses of incident HF, cases of prevalent HF (n=30) were excluded. For analyses of incident CE, prevalent cases of CAD (n=185) and HF (n=30) were excluded prior to analysis. A total of 590 subjects departed from ACM and of these n=353 from

CVM with a median FUT 12.7 years. One-hundred-and-thirty subjects developed incident HF (median FUT 10.8 years) and 189 subjects developed incident CE (median FUT 10.7 years). A flowchart of the study population of **paper I** and **II** is provided in **Figure 3.**

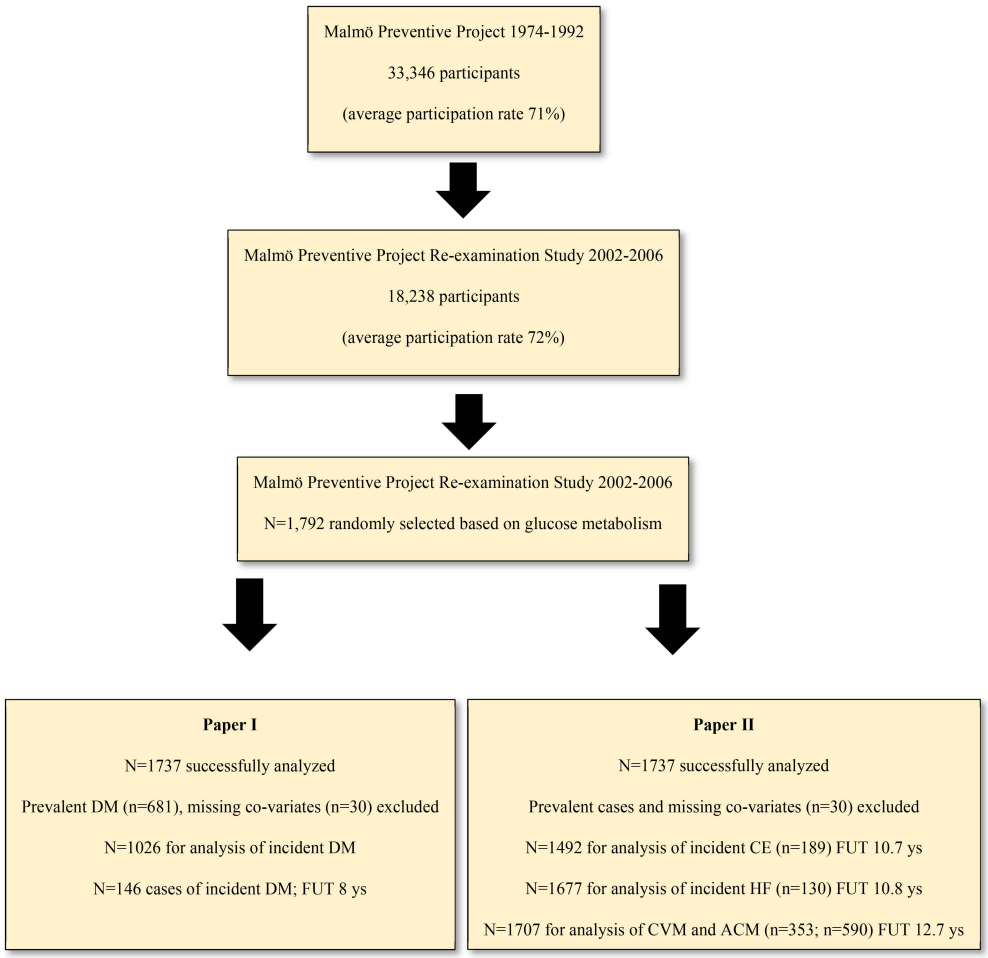

Figure 3. Flowchart of MPP in paper I and II.
DM diabetes mellitus; FUT median follow-up time; ys years; CE coronary events; HF heart failure; CVM cardiovascular mortality; ACM all-cause mortality

Paper III

In Paper III we examined NT-proBNP, mid-regional proadrenomedullin (MR-proADM), copeptin, C-terminal pro-endothelin-1 (CT-pro-ET-1) and cystatin C and their prognostic role regarding ACM and re-hospitalization for cardiac causes in an acute HF setting. Between March 2014 and October 2017, a total of 283 consecutive patients hospitalized for HF were included in HARVEST-Malmö and underwent clinical examination including transthoracic echocardiography (TTE). Of these, 268 patients had a complete dataset on all covariates and were included in the study. A total of 57 subjects died with a median FUT of 17 months, and 90 patients were re-hospitalized due to cardiac causes (median FUT 5 months). A flowchart of the study population of **paper III** and **IV** is provided in **Figure 4.**

Paper IV

In Paper IV we investigated the diagnostic and prognostic abilities of Proenkephalin A 119-159 (penKid) and bioactive adrenomedullin (bio-ADM) in two European HF cohorts. In both cohorts, patients were examined for signs of congestion (dyspnoea, edema, signs of congestion on X-ray and auscultatory lung rales) and a clinical congestion score (CCS) was calculated by summing the individual scores for each sign of congestion. Worsening renal function was defined as an increase of plasma creatinine of >26.5 μmol/L or 0.3 mg/dL or 50% higher than the admission value within 48 hours of admission as used in previous studies(69, 70).

Between March 2014 and August 2018, 324 consecutive patients were included in HARVEST-Malmö for acute HF.

Between May 2013 and March 2015, 245 patients that received a final diagnosis of HF were enrolled to the GREAT Network Acute Heart Failure Rome Study. For all analyses subjects with missing data on any co-variates were excluded leaving a total of 530 subjects examined for congestion, worsening renal function, in-hospital mortality and length of hospital stay. Since there was no follow-up data from patients included from the GREAT Rome Study analyses of 1-year mortality and re-hospitalization were only performed in subjects included from HARVEST-Malmö. A flowchart of the study population of **paper III** and **IV** is provided in **Figure 4.**

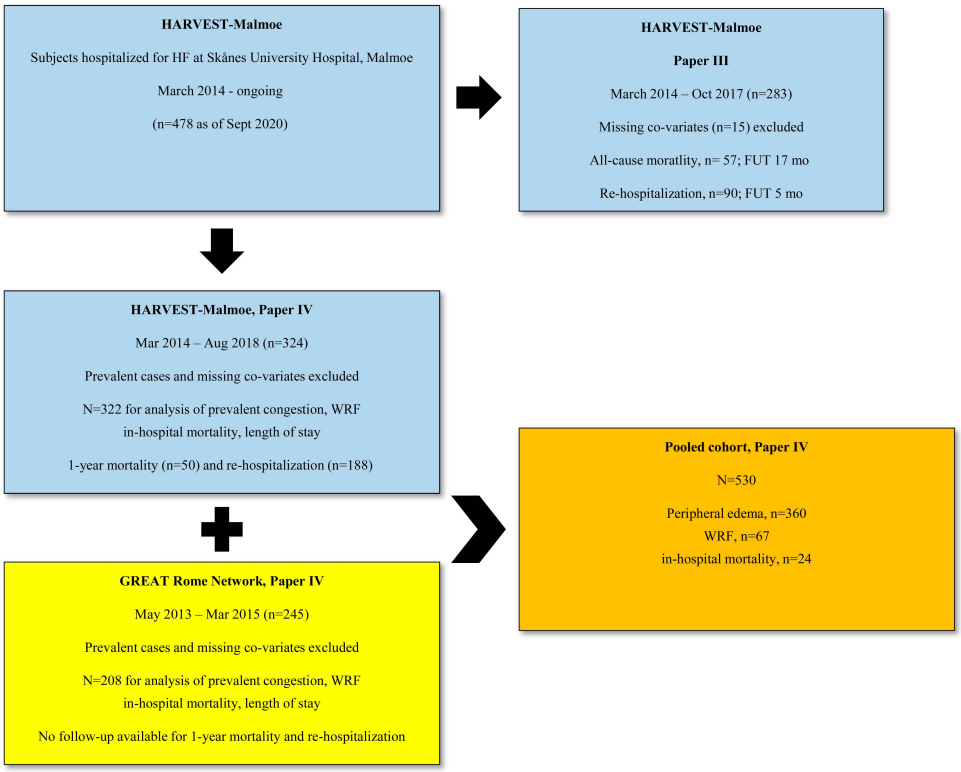

Figure 4. Flowchart of HARVEST-Malmö and GREAT Rome Network cohort in paper III and IV.
FUT median follow-up time; mo months; WRF worsening renal function

Description of variables

Prevalent and incident DM

In **paper I** and **II** prevalent DM at baseline was defined as a self-reported physician diagnosis of DM, use of antidiabetic medication, a diagnosis of DM in any of the local or national diabetes registries prior to study entry, or two separate FPG measurements of ≥7.0 mmol/L when available. In **paper III** and **paper IV** prevalent DM was defined as either self-reported physician diagnosis of DM, use of antidiabetic medication or FPG ≥7 mmol/L. In **paper I,** data regarding incident DM was retrieved through record linkage of the Swedish personal identification number with national and regional registries as follows: The Malmö HbA1c Register that analyzed all HbA1c samples at the Department of Clinical Chemistry obtained in institutional and non-institutional care in Malmö from 1988 and onwards(71); The Swedish National Diabetes Register(72); The Regional Diabetes 2000 Register of the Skåne Region(73); The Swedish National Patient Register covering all somatic and psychiatric hospital

discharges and hospital based outpatient care(74); The Swedish Cause-of-Death Register(75); and The Swedish Prescribed Drug Register (prescription of anti-diabetic medication)(76). Type of diabetes was not specified from all registries but given the mean age of the study population and since all prevalent cases of diabetes were excluded, it is reasonable to assume that an absolutely overwhelming majority of the incident cases of diabetes were type 2 diabetes (DM).

Hypertension

In **paper I** and **paper II,** hypertension was defined as systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg or the use of anti-hypertensive medication including calcium-channel blockers, beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, long acting nitrates and diuretics. Heart rate and blood pressure were measured twice in the supine position after 10 min of rest. In **paper III** and **paper IV** hypertension was defined as SBP >140 or DBP >90 mmHg. In HARVEST-Malmö nurses measured BP using a validated automated monitor and in MPP-RES echo an automated sphygmomanometer was used.

Smoking

Data on smoking was self-reported in all cohorts.

Prevalent and incident CVD

In **paper II,** prevalent HF was defined as a self-reported diagnosis of HF and by means of International Classification of Diseases (ICD) codes acquired from the local hospital diagnosis registry was used to define prevalent HF (ICD-10 code I50). Diagnoses of prevalent CE were self-reported and also by retrieval from local hospital diagnosis registry (ICD-10 codes I21, I22, I25). For incident HF and CE, participants were followed in local and national registers. CE was defined as coronary revascularization and/or fatal or nonfatal myocardial infarction. Data on ACM and CVM were retrieved through the Swedish Board on Health and Welfare and Statistics Sweden. Follow-up ended on 31st December 2018. Diagnoses of incident CE and HF were retrieved from record linkage using the Swedish personal identification number with the Swedish Hospital Discharge Register (SNHDR), the Swedish Cause of Death Register, and the Swedish Coronary Angiography and Angioplasty Registry.

In **paper III** and **paper IV** data on ACM was retrieved from the Swedish National Board of Health and Welfare's Cause of Death Register. Data regarding the in-hospital mortality and re-hospitalization due to cardiac causes were retrieved from the individual electronic medical records of the Skåne Health Care Region (Melior, Siemens Health Services, Solna, Sweden).

In **paper IV** prevalent HF was defined as either prior hospitalisation for HF or a HF diagnosis prior to inclusion in the study.

Laboratory tests

All fasting analyses were performed at the Department of Clinical Chemistry, Skåne University Hospital, attached to a national standardization and quality control system.

Biomarkers

For MPP-RES echo and HARVEST-Malmö biomarkers were analysed from fasting plasma samples frozen at -80°C. In the GREAT Rome study blood samples were collected at admission and stored at -80°C.

Proteomic analysis

In **paper I** and **II** plasma levels of 92 proteins were analyzed by the Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD III 96 × 96 reagents kit (Olink Bioscience, Uppsala, Sweden) which uses two oligonucleotide-labeled highly specific antibodies to bind to each target protein, which allows the formation of a polymerase chain reaction sequence that can then be detected and quantified (60). All data are presented as arbitrary units. One protein (NT-proBNP) was below detectable limits in >15% samples. Mean intra-assay and inter-assay variations were observed to be 8.1% and 11.4%, respectively.

NT-proBNP

In **paper II** NT-proBNP was measured with an electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics, Basel, Switzerland) at the Department of Clinical Chemistry, Akershus University Hospital, Lorenskog, Norway. In **paper III** and **IV** NT-proBNP was analysed at the Department of Clinical Chemistry, Skåne University Hospital in Malmö, participating in a national standardization and quality control system using a sandwich assay based on ElectroChemiLuminiscence Immunoassay (Cobas, Roche Diagnostic, Basel, Switzerland). In **paper IV,** in the GREAT Rome study BNP and NT-proBNP were measured in Architect assays (Abbott Laboratories Diagnostics Division, Abbott Park, IL 60064 USA).

Cystatin C

Cystatin C was analysed at the Department of Clinical Chemistry, Skåne University Hospital, using an automated particle-based immunoassay (Hitachi Modular P analysis system; Roche, Basel, Switzerland).

Copeptin

Copeptin was measured at baseline using an ultrasensitive assay on KRYPTOR Compact Plus analyzers and a commercial sandwich immunoluminometric assay(77) (Thermo Fisher Scientific,B.R.A.H.M.S Biomarkers)

Mid-regional pro-adrenomedullin (MR-proADM)

MR-proADM levels were analysed via specific sandwich immunoluminometric assays (KRYPTOR, B.R.A.H.M. S, Berlin, Germany) in EDTA-treated plasma (78).

C-terminal pro-endothelin-1 (CT-pro-ET-1)

C-terminal pro-endothelin-1 was measured at baseline using Thermo Fisher Scientific B.R.A.H.M.S CT-pro-ET-1 KRYPTOR (79).

Proenkephalin A 119-159 (PenKid)

PenKid was measured by a chemiluminescence immunoassay (Sphingotest® penKid®, Sphingotec GmbH, Hennigsdorf, Germany) (80).

Bioactive adrenomedullin (bio-ADM)

Bio-ADM is the biologically form of adrenomedullin and was measured using a chemiluminescence immunoassay (Sphingotest® bio-ADM®, Sphingotec GmbH, Hennigsdorf, Germany)(81).

Echocardiography

In **paper III** TTEs were obtained by experienced sonographers using a Philips IE33 (Philips, Andover, MA, USA) with a 1–5 MHz transducer (S5-1), or a GE Vingmed Vivid 7 Ultrasound (GE, Vingmed Ultrasound, Horten, Norway) with a 1–4 MHz transducer (M3S). Measurements of ejection fraction, left ventricular volumes, atrial volumes, wall thickness were performed offline using Xcelera 4.1.1 (Philips Medical Systems, The Netherlands) according to the recommendations of the American Society of Echocardiography (82). Left ventricular mass was calculated according to the Devereux formula (83). The values were indexed to body surface area. Cine loops were obtained from standard views (parasternal long axis, apical 4- and 2-chamber). Internal left and right ventricular dimensions were measured from parasternal long axis view at end-diastole. Measurements of wall thickness were obtained in two-dimensional end-diastolic parasternal long axis view. Left ventricular volumes were calculated using the biplane Simpson method of disks, by manual tracing (papillary muscles included in the cavity) in two-dimensional end-diastolic and end-systolic frames defined as the largest and smallest left ventricular cavities, respectively,

in apical 4- and 2-chamber projections. Ejection fraction (EF) was calculated automatically from end-diastolic volumes (EDV) and end-systolic volume (ESV) using the following formula: EF=(EDV-ESV)/EDV.

Statistical analysis

Paper I

Non-normally distributed variables were ln-transformed prior to analysis. The analyzed proteins were subsequently standardized by z-score transformation. Cox regression models and Harrell's concordance index (C-index)(84) were used to calculate hazard ratios (HR) for incident DM per standard deviation (SD) of change of ln-transformed values in age- and sex-adjusted models (model 1). Proportional hazard assumption was tested using Schoenfeld residuals. Bonferroni-correction was used to address the issue of multiple testing *(85)*. Only proteins that remained significant after Bonferroni correction ($0.05/91 = 5.5 \times 10^{-4}$) in model 1 *(age and sex)* were further tested in the multivariable Cox regression model and Harrell's C-index (model 2), which was adjusted for age, sex, BMI, hypertension, antihypertensive treatment, TG, HDL, cystatin C and physical activity. Furthermore, FPG was included on top of Model 2. The proteins associated with incident DM in model 1 were also tested for association with prevalent DM using logistic regressions in models 1, 2 and 3. All analyses were carried out using SPSS 22 (IBM, Armonk, New York, USA).

Paper II

All seven analysed proteins were ln-transformed and then standardized by z-score transformation. The proportional hazards assumption was tested using partial residuals. Cox regression was carried out adjusted for age and sex (Model 1), and a Bonferroni-corrected p-value < 0.007 (0.05/7) was considered statistically significant. Proteins that were significantly associated with the outcome were further analyzed using models adjusted for other relevant co-variates, in which a p-value <0.05 was considered significant. All analyses were adjusted for age, sex, BMI, smoking, DM, SBP, antihypertensive treatment (AHT), prevalent atrial fibrillation and cystatin C. For the analysis of ACM and prevalent CVD, prevalent HF, total cholesterol and HDL were included (Model 2a). For the analysis of incident CE; total cholesterol and HDL were included, and prevalent cases of CVD and HF were excluded prior to analysis (Model 2a). For the analysis of HF, prevalent CVD, heart rate and NT-proBNP were included and prevalent cases of HF excluded (Model 2b). All analyses were carried out using SPSS 25.

Paper III

All five investigated biomarkers were log-transformed and standardized by z-score transformation. Multivariable-adjusted Cox regression analyses were performed in two different models. Model 1 included age and sex, whereas Model 2 included age, sex, BMI, DM, smoking, atrial fibrillation, SBP, total cholesterol, HDL, and

NYHA-class at admission. Follow-up time was calculated as time between screening date and date of the first re-hospitalization, death, or end of follow-up through 1 October 2017. All analyses were performed using IBM SPSS 23, and a two-sided Bonferroni-corrected P-value of 0.05/5 = 0.010 was considered statistically significant in the Cox regression analyses. Echocardiographic measurements (eight different parameters) were analysed for associations with the five biomarkers in age-adjusted and sex-adjusted linear regression analysis, and thus a two-sided Bonferroni-corrected p-value <0.05/13 = 0.0038 was considered statistically significant.

Paper IV

In both cohorts both bio-ADM and penKid were log-transformed and standardized by z-score transformation prior to analyses. Since different assays were used for BNP/Nt-proBNP, the data from both cohorts were log transformed and then normalised (using z-score transformation) prior to pooling of the natriuretic peptide data. For all analyses, subjects with missing data on any of the covariates were excluded. The area under the curve (AUC) of bio-ADM for peripheral oedema, in-hospital mortality, 1-year mortality and rehospitalisation was calculated by receiver operating characteristic (ROC) analysis. As for penKid, AUC was calculated for worsening renal function (WRF) and in-hospital mortality. The cross-sectional associations of bio-ADM with each of the four signs of congestion, as well as bio-ADM with a clinical congestion score, were explored using logistic regression models. Correlations between penKid and creatinine on admission were explored using Spearman's correlation test. The cross-sectional associations of penKid and WRF were explored using two logistic regression models: crude (univariable) and in the pooled cohort adjusted for DM, SBP, angiotensin converting enzyme inhibitor (ACEi), angiotensin receptor blocker (ARB), betablockers, HF, creatinine and BNP; all previously associated with WRF (multivariable). The cross-sectional associations of bio-ADM and penKid with in-hospital mortality were explored using uni-variable and bi-variable logistic regression models due to the low event rate (24 events in the pooled cohort). In HARVEST-Malmö, additional analyses were carried out for 1-year mortality and rehospitalization at follow-up, using Cox regression models. In analyses of re-hospitalization, subjects that deceased during hospital stay were excluded from the model prior to analysis. Analyses of length of hospital stay were obtained using linear regression models. Analyses were performed using IBM SPSS 25 and a two-sided $p<0.05$ was considered statistically significant.

Results

Paper I

Subjects with prevalent DM at baseline (n=681) had higher TG and lower HDL levels, higher BMI, increased prevalence of hypertension and worse renal function. Subjects with incident diabetes (n=146; median follow-up time 8.0 years; interquartile range 12 years) were more often male, had higher BP, higher TG and lower HDL levels, as well as higher BMI at baseline, compared with those who did not develop diabetes. Baseline characteristics for subjects with and without prevalent DM are presented in **Table 1** and baseline characteristics for subjects with and without incident DM are presented in **Table 2**.

Table 1. Baseline Characteristics of Study Participants with and without Prevalent Diabetes

	All subjects (n=1707)	Subjects without prevalent DM (n=1026)	Subjects with prevalent DM (n=681)	p-value
Age (years)	67.4 (±6.0)	66.9 (±6.1)	68.1 (±5.9)	<0.001
Sex (% female)	498 (29.1)	331 (32.2)	167 (24.4)	0.001
BMI	28.3 (±4.3)	27.4 (±3.9)	29.8 (±4.6)	<0.001
SBP (mmHg)	146.9 (±19.8)	145.3 (±19.2)	149.1 (±20.6)	<0.001
HT (%)	1069 (62.6)	570 (55.6)	499 (73.3)	<0.001
FPG (mmol/l)	6.2 (5.6-7.4)	5.8 (5.3-6.2)	7.8 (7.1-9.2)	<0.001
TG (mmol/l)	1.3 (0.9-1.8)	1.1 (0.8-1.6)	1.5 (1.0-2.0)	<0.001
HDL (mmol/l)	1.3 (1.0-1.5)	1.3 (1.1-1.6)	1.2 (1.0-1.4)	<0.001
Cystatin C (mg/l)	1.06 (0.95-1.20)	1.05 (0.95-1.19)	1.08 (0.95-1.24)	0.002

BMI; body mass index, SBP; systolic blood pressure; DBP; diastolic blood pressure, HT; hypertension, FPG; fasting plasma glucose, TG; triglycerides, HDL; high-density lipoprotein cholesterol. Values are displayed as means (±standard deviation) or, for skewed variables, medians and interquartile (25-75) range

Table 2 Baseline Characteristics of Study Participants with and without Incident Diabetes

	All subjects (n=1026)	Subjects without incident diabetes (n=880)	Subjects with incident diabetes (n=146)	p-value
Age (years)	66.9 (±6.1)	66.9 (±6.1)	66.7 (±5.8)	0.071
Sex (% female)	331 (32.2)	290 (32.8)	41 (28.1)	0.024
BMI	27.4 (±3.9)	27.0 (±3.7)	29.4 (±4.1)	<0.001
SBP (mmHg)	145.3 (±19.2)	145.3 (±19.5)	145.6 (±17.0)	0.002
HT (%)	571 (55.6)	467 (53.0)	104 (71.2)	0.002
FPG (mmol/l)	5.8 (5.3-6.2)	5.7 (5.3-6.2)	6.3 (6.1-6.6)	<0.001
TG (mmol/l)	1.1 (0.8-1.6)	1.1 (0.8-1.5)	1.3 (1.0-1.7)	<0.001
HDL (mmol/l)	1.3 (1.1-1.6)	1.4 (1.1-1.6)	1.2 (1.0-1.4)	<0.001
Cystatin C(mg/l)	1.05 (0.95-1.19)	1.01 (0.95-1.18)	1.08 (0.95-1.22)	0.114

BMI; body mass index, SBP; systolic blood pressure; FPG; fasting plasma glucose, HT; hypertension, TG; triglycerides, HDL; high-density lipoprotein cholesterol. Values are displayed as means (± standard deviation) or, for skewed variables, medians and interquartile (25-75) range.

Associations of proteins with incident diabetes

In age- and sex-adjusted Cox analyses (model 1), seven proteins were associated with incident DM and fulfilled the pre-specified Bonferroni-corrected p-value of $<5.5x10^{-4}$: paraoxonase-3 (PON3), fatty acid binding protein −4 (FABP4) , plasminogen activator inhibitor 1 (PAI-1), insulin-like growth factor-binding protein 2 (IGFBP-2), scavenger receptor cysteine rich type 1 protein M130 (CD163), cathepsin D (CatD) and Galectin-4 (Gal-4). Results from the fully adjusted Cox regression analyses (models 2 and 3) are presented in **Table 3.**

Table 3. Cox Regression Analysis Examining Proteins relation to Incident Diabetes

	Model 1		Model 2		Model 3	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
PON 3	0.65 (0.56-0.75)	3.3x10-9	0.79 (0.67-0.93)	0.005	0.81 (0.69-0.96)	0.014
FABP4	1.74 (1.44-2.10)	9.3x10-9	1.46 (1.16-1.84)	0.001	1.48 (1.17-1.87)	0.001
PAI-1	1.70 (1.41-2-05)	4.0x10-8	1.50 (1.21-1.84)	<0.0001	1.40 (1.14-1.72)	0.001
IGFBP-2	0.66 (0.56-0.77)	2.9x10-7	0.82 (0.68-0.99)	0.039	0.89 (0.74-1.08)	0.24
CD163	1.50 (1.26-1.77)	3.0x10-6	1.34 (1.11-1.60)	0.002	1.22 (1.02-1.46)	0.029
CatD	1.33 (1.13-1.56)	5.2x10-4	1.20 (1.00-1.43)	0.050	1.08 (0.91-1.29)	0.39
Gal-4	1.37 (1.15-1.64)	5.4x10-4	1.30 (1.08-1.56)	0.005	1.27 (1.07-1.52)	0.008

Cox regression for incident diabetes (146 cases vs. 880 controls)) adjusted for age and sex (Model 1) and age, sex, BMI, HTN, TG, HDL, cystatin C and physical activity (Model 2) and age, sex, BMI, HTN, TG, HDL, cystatin C, physical activity and fasting plasma glucose (Model 3). PON 3; Paraoxonase, FABP4; fatty acid binding protein 4, PAI-1; Plasminogen activator inhibitor 1, IGFBP-2; Insulin-like growth factor-binding protein 2, CD163; scavenger receptor cysteine rich type 1 protein M130, CatD; Cathepsin D, Gal-4; Galectin-4.

Associations of proteins with prevalent diabetes

All seven proteins associated with incident DM in model 1 were significantly associated (p-values $< 5.5x10^{-4}$) with prevalent diabetes in a binary logistic regression model 1. However, in the fully adjusted model 3 only Gal-4 and PAI-1 were significantly associated with prevalent diabetes.

Harrell's concordance index models

None of the proteins showed a substantial increase in C-index.

Paper II

Baseline characteristics of all subjects (n=1713), those deceased (n=590), with incident CE (n=189), and with incident HF (n=130) are presented in **Table 4**. The overall study population had a mean age of 67.4 years. More than two thirds of the population were male and more than a third had prevalent diabetes at baseline. There were no interactions between the investigated proteins and diabetes in the endpoint analyses.

Table 4. Baseline characteristics of the study population

	All subjects (n=1713)	**Deceased** (n=590)	**Incident HF** (n=130)	**Incident CE** (n=189)
Demographics				
Age (years)	67.4 (±6.0)	70.9 (±4.9)	70.4 (±4.8)	69.0 (±5.6)
Women, n (%)	498 (29.1)	179 (30.3)	28 (21.5)	28 (14.8)
Smoking, n (%)	303 (17.7)	125 (21.2)	23 (17.7)	32 (16.9)
Clinical profile				
BMI (kg/m2)	28.3 (±4.3)	28.5 (±4.6)	29.5 (±5.0)	28.0 (±3.9)
Systolic BP (mmHg)	146.6 (±20.2)	146.5 (±21.1)	148.2 (±21.5)	151.6 (±20.8)
Heart rate (BPM)	71.8 (±12.5)	72.0 (12.5)	70.5 (±14.4)	71.1 (±12.5)
Medical history				
Diabetes, n(%)	604 (35.3)	298 (50.5)	72 (55.4)	88 (46.6)
Prevalent HF, n (%)	30 (1.8)	25 (4.3)	N/A	N/A
Prevalent AF, n (%)	97 (5.7)	65 (11.0)	16 (12.3)	13 (6.9)
Prevalent CVD, n (%)	185 (10.8)	100 (16.9)	41 (31.5)	N/A
AHT, n (%)	802 (46.8)	348 (59.0)	86 (66.2)	104 (55.0)
Laboratory				
Cystatin C (mg/L)	1.06 (0.95-1.20)	1.14(0.99-1.33)	1.16(1.0-1.3)	1.12(0.99.1.29)
NT-proBNP (pg/mL)	12 (6-25)	21 (10-45)	32 (15-69)	14 (7-33)
Cholesterol (mmol/L)	5.40 (4.6-6.2)	5.2 (4.4-6.0)	5.0 (4.1-5.7)	5.5 (4.7-6.2)
HDL-C (mmol/L)	1.25 (1.03-1.52)	1.24 (1.03-1.50)	1.20 (1.0-1.4)	1.22 (0.97-1.43)

Values are displayed as means (±standard deviation) or, for skewed variables, medians and interquartile (25-75) range. AF; atrial fibrillation; BMI; body mass index, BPM; beats per minute; BP; blood pressure; AHT; anti-hypertensive treatment; CVD; cardiovascular disease; HF; heart failure. NT-proBNP; N-terminal pro-B-type natriuretic peptide; HDL high-density lipoprotein; N/A not applicable, excluded prior to analysis

Results from the fully adjusted models for all endpoints can be found in **Table 5.**

Analyses of all-cause mortality (ACM)

Five of the seven proteins (Gal-4, CatD, IGFBP2, CD163 and FABP4) were significantly associated with ACM in model 1 and model 2a (median follow-up time 12.7 years, interquartile range (IQR): 11.2-13.6 years; 590 deaths; **Table 5**).

Analyses of cardiovascular mortality (CVM)

Five proteins (Gal-4, CatD, IGFBP2, CD163 and FABP4) yielded significant associations with CVM in age and sex adjusted Cox regression analyses, (median follow-up time 12.7 years, IQR 11.2-13.6 years; 353 deaths). After further adjustment according to Model 2a, all but FABP4 remained significantly associated with CVM (**Table 5)**.

Analyses of incident coronary events (CE)

Three proteins (Gal-4, CatD, FABP4) yielded significant associations with incident CE (median follow-up time 10.7 years, IQR 10.0-11.7 years; 164 events) in both model 1 and model 2a (**Table 5**).

Analyses of incident heart failure (HF)

Four proteins (Gal-4, CatD, FABP4, PON3) yielded significant associations with incident HF in age and sex adjusted Cox regression analyses (median follow-up time 10.8 years, IQR 10.2-11.7; 105 events), but only Gal-4 and CatD remained significantly associated with incident HF after further adjustment for Model 2b (**Table 5**).

Table 5. Cox Regression Analyses for risk of All-Cause Mortality, Cardiovascular mortality, incident Heart failure and incident Coronary Events

	All-Cause Mortality			Cardiovascular Mortality			Incident Heart failure			Incident Coronary Events		
	Model 2a			Model 2a			Model 2b			Model 2a		
	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Gal-4	1.29	1.17-1.41	1.9x10-7	1.38	1.22-1.56	4.8x10-7	1.26	1.03-1.54	0.024	1.34	1.14-1.57	0.0004
CatD	1.26	1.15-1.37	2.1x10-7	1.28	1.14-1.43	6.7x10-5	1.21	1.01-1.46	0.044	1.30	1.12-1.50	0.001
FABP4	1.16	1.04-1.45	0.010	1.14	0.99-1.32	0.065	0.95	0.75-1.19	0.64	1.27	1.05-1.55	0.015
CD163	1.19	1.09-1.29	0.00009	1.21	1.09-1.36	0.001	*	*	*	*	*	*
IGFBP2	1.17	1.05-1.30	0.004	1.18	1.03-1.35	0.020	*	*	*	*	*	*
PON3	*	*	*	*	*	*	0.95	0.81-1.12	0.57	*	*	*
PAI-1	*	*	*	*	*	*	*	*	*	*	*	*

* Did not reach Bonferroni-corrected p-value of 0.007 (0.05/7) in age- and sex adjusted model 1 and was therefor not further analysed in fully adjusted models.
Model 2a and Model 2b is adjusted for age, sex, BMI, systolic BP, AHT, smoking, DM, atrial fibrillation, prevalent CVD, cystatin C. **Model 2a** is further adjusted for prevalent HF, total cholesterol and HDL. In **Model 2b** prevalent cases of HF were excluded prior to analysis and model was further adjusted for heart rate and NT-proBNP.

BMI body mass index; BP blood pressure; AHT antihypertensive treatment; CVD cardiovascular disease. Gal-4 galectin-4; CatD cathepsin D; FABP4 fatty acid binding protein 4; CD163 Scavenger receptor cysteine rich type 1 protein M130; IGFBP2 Insulin-like growth factor-binding protein 2; PON3 Paraoxonase-3; PAI-1 Plasminogen activator inhibitor 1

Paper III

Baseline characteristics of the study population (n=268) are presented in **Table 6**. The mean age was 75 years, subjects were predominantly male (71%) and 39% had DM. More than 90% were treated with beta-blockers and ACEi/ARB.

A total of 57 subjects died during follow-up period (median time, 17 months; IQR 8–29).The most frequent cause of death was HF (n = 21) followed by sudden cardiac death (n = 7), cancer (n = 2), and stroke (n = 2). The remaining death causes (n = 21) consisted of different diagnoses and were defined as 'other' in the database.

During follow-up (median 5 months; IQR 1-12) 90 patients were re-hospitalized because of cardiac causes with the dominant cause being HF (n=79) followed by cardiac arrhythmia (n = 10) and myocardial infarction (n = 1).

Table 6. Baseline characteristics of the study population, HARVEST-Malmö (n=268)

Age (years)	75.1 (±11.0)
Sex (female n;(%))	77 (29)
Smoking (n; (%))	31 (11.6)
BMI (kg/m2)	27.4 (±5.6)
SBP (mmHg)	137.4 (±27.7)
DBP (mmHg)	79.2 (±15.3)
HT (n; (%))	106 (39.6)
Diabetes (n; (%))	105 (39)
AF (n; (%))	157 (58.6)
Newly diagnosed HF (n, (%))	85 (32)
LVEF (%)	39.1 (16.2)
Loop-diuretics (n, (%))	258 (96)
B-blockers (n, (%))	137 (92)
ACEi or ARB (n, (%))	208 (78)
HDL (mmol/L)	1.2 (0.4)
Cholesterol (mmol/L)	3.6 (1.1)
GFR (ml/min)	45.9 (16.8)
Nt-proBNP (pmol/L)	4077.5 [2175.0-8125.8]
Cystatin C	1.6 [1.3-2.1]
Copeptin	30.9 [14.7-49.2]
MR-proADM	1.6 [1.1-2.2]
CT-proET1	149.3 [118.9-200.0]

Values are means (±standard deviation (SD) or median [25th-75th interquartile range]. BMI=body mass index; SBP= systolic blood pressure ; DBP= diastolic blood pressure; HT=hypertension; ACEi=angiotensin converting enzyme inhibitors; ARB= angiotensin II receptor antagonists; LVEF= LV ejection fraction; AF=atrial fibrillation, GFR=glomerular filtration rate.

Biomarkers and mortality

In Cox regression analyses adjusted for age and sex (*model 1)*, all biomarkers except CT-pro-ET1, were significantly associated with increased post-discharge mortality (NT-proBNP, copeptin, MR-proADM, cystatin C; **Table 7**). In *model 2*, all biomarkers except CT-pro-ET1 were significantly associated with mortality; **Table 7**.

Table 7. Biomarkers and risk of all-cause mortality

All-cause mortality (n=57)		
	HR (95 CI%)	p-value
Cystatin C		
Model 1	1.99 (1.52-2.62)	5.8x10-7
Model 2	2.11 (1.56-2.86)	1.0x10-6
NT-proBNP		
Model 1	1.88 (1.37-2.57)	8.2x10-5
Model 2	1.85 (1.32-2.61)	4.0x10-4
Copeptin		
Model 1	1.63 (1.20-2.20)	0.002
Model 2	1.70 (1.22-2.36)	0.002
MR-proADM		
Model 1	1.78 (1.32-2.41)	1.9x10-4
Model 2	1.94 (1.36-2.75)	2.2x10-4
CT-proET1		
Model 1	1.45 (1.08-1.95)	0.014
Model 2	1.42 (1.03-1.95)	0.034

Model 1: adjusted for age and sex

Model 2: adjusted for age, sex, body mass index, diabetes, smoking, atrial fibrillation, systolic blood pressure, total cholesterol, high density lipoprotein and NYHA-class at admission

Biomarkers and re-hospitalization

In *model 1,* cystatin C and NT-proBNP were the only two of the five biomarkers significantly associated with risk of re-hospitalizations due to cardiac causes. In the fully adjusted *model 2*, NT-proBNP was the only biomarker that showed Bonferroni-adjusted significant association with risk of re-hospitalization due to cardiac causes (**Table 8**).

Table 8. Biomarkers and Risk of Re-hospitalization

1st Re-hospitalization (n=90)		
	HR (CI 95%)	p-value
Cystatin C		
Model 1	1.33 (1.08-1.65)	0.008
Model 2	1.27 (1.01-1.59)	0.040
Nt-proBNP		
Model 1	1.39 (1.10-1.77)	0.007
Model 2	1.43 (1.10-1.87)	0.009
Copeptin		
Model 1	1.20 (0.96-1.49)	0.115
Model 2	1.20 (0.94-1.53)	0.152

Model 1: adjusted for age and sex

Model 2: adjusted for age, sex, body mass index, diabetes, smoking, atrial fibrillation, systolic blood pressure at admission, total cholesterol, high density lipoprotein and NYHA-class at admission

Biomarkers and echocardiographic measurements

In age and sex adjusted linear regression models, NT-proBNP was robustly associated with reduced ejection fraction (β -7.07, $p=8.6x10^{-10}$). MR-proADM (β 1.67, p=0.001) and CT-proET1 (β 1.45, p=0.002) were significantly associated with increased right ventricular size. Finally, high levels of cystatin C were significantly associated with left posterior left ventricular wall hypertrophy (β 1.81, p=0.001).

Paper IV

Baseline characteristics for the HARVEST-Malmö cohort (n=322), GREAT Rome cohort (n=208) and the pooled cohort (n=530) are presented in **Table 9.** In the pooled cohort, 63.4% of the subjects presented with peripheral edema as a sign of congestion; 14.3% had worsening renal function; the median length of stay was 6 days, and the in-hospital mortality reached 5.1%. In the HARVEST-Malmö cohort where we had access to follow-up data, the re-hospitalization rate was 66.2% (median follow-up 174 days) and the 1-year mortality was 17.2%.

Table 9. Baseline characteristics of the HARVEST-Malmö cohort, the GREAT Network Rome cohort and the pooled cohort

	HARVEST–Malmö n=322	GREAT Rome n=208	Pooled cohort n=530	p-value
Demographics, n (%)				
Age (years)	75.1 +11.1	78.5 +9.9	76.4 +10.7	<0.001
Female sex (%)	97 (30.1)	114 (54.8)	211 (39.8)	<0.001
Smoking (%)	38 (11.8)	29 (13.9)	67 (12.6)	0.450
Clinical profile, n (%)				
Systolic blood pressure (mmHg)	137.3 +27.5	150.7 +33.7	142.3 +30.8	<0.001
Diastolic blood pressure (mmHg)	73.6 +12.6	81.4 +16.8	77.0 +14.9	<0.001
Length of stay (days)	7 (4-9)	6 (3-8)	6 (4-9)	<0.001
Ejection fraction (%)	38 (24-52)	40 (27-50)	40 (25-50)	0.036
Worsening renal function (%)	30 (9.3)	37 (17.8)	67 (12.6)	<0.001
Persistent dyspnoea at admission	295 (91.6)	191 (91.8)	486 (91.7)	0.980
Edema	215 (66.8)	145 (69.8)	360 (67.9)	0.004
Signs of congestion on CXR	261 (81.1)	181 (87.0)	442 (83.4)	0.590
Rales at auscultation	223 (69.3)	171 (82.2)	394 (75.7)	0.002
Medical history, n (%)				
Diabetes mellitus	119 (37.0)	75 (36.1)	194 (36.6)	0.720
Prior heart failure	200 (62.1)	85 (40.9)	285 (53.8)	<0.001
Prevalent atrial fibrillation	153 (47.5)	96 (46.2)	249 (47.0)	0.762
Medication, n (%)				
ACE inhibitors/ARB	251 (77.9)	109 (52.4)	360 (67.9)	<0.001
Beta-blockers	279 (86.6)	101 (48.6)	380 (71.7)	<0.001
Diuretics	306 (95.0)	132 (63.5)	438 (82.6)	<0.001
Laboratory				
Bio-ADM (pg/mL)	39.6 (25.6-64.5)	24.6 (9.5- 48.4)	34.6 (18.7-59.3)	<0.001
penKid (pmol/L)	85.3 (62.8-118.4)	109.5 (81.7-168.5)	91.8 (67.9-135.9)	<0.001
BNP (pg/mL)	-	756 (366-1452)	-	-
NT-proBNP (pg/mL)	4096 (2212-8645)	7811 (2038-10851)	-	-
Creatinine (mg/dL) admission	1.32 +0.64	1.44 +0.91	1.37 +0.76	0.069
Creatinine (mg/dL) after 48 hours	1.35 +0.64	1.53 +1.0	1.42 +0.81	0.016
Potassium (mmol/L)	3.8 +0.5	4.3 +0.6	4.0 +0.6	<0.001
Sodium (mmol/L)	140.5 +3.3	137.3 +5.9	139.2 +4.7	<0.001
Haemoglobin (g/L)	127.6 +18.3	123.2 +24.8	125.9 +21.2	0.019
Outcomes				
Peripheral edema (n;(%))	215 (66.8)	145 (69.8)	360 (67.9)	0.004
WRF (n;(%))	30 (8.1)	37 (20.7)	67 (14.3)	<0.001
In-hospital mortality (n;(%))	7 (1.9)	17 (8.2)	24 (5.1)	<0.001
One-year mortality (n;(%))	50 (17.2)	-	-	-

Continuous data presented as mean±standard deviation or median (Q1–Q3), depending on distribution. CXR, chest X-ray; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP; bio-ADM, bioactive adrenomedullin; penKid, proenkephalin A 119-159

Congestion

Logistic regression analyses carried out for bio-ADM and each sign of congestion (dyspnoea, peripheral edema, signs of congestion on chest X-ray, and lung rales upon auscultation), revealed that each one SD increment of bio-ADM was only significantly associated with peripheral edema and no other sign of congestion. Bio-ADM remained significantly associated with peripheral edema with an odds ratio of 2.30 (1.29-2.97); p-value <0.001 even after adjusting for prior HF, systolic BP, Nt-proBNP/BNP and atrial fibrillation in the pooled cohort.

Analyses of bio-ADM and severe congestion (congestion score of 4) revealed significant association between bio-ADM and risk of having the highest congestion score; however, that association was driven solely by the association of bio-ADM and peripheral edema.

Worsening renal function (WRF)

PenKid was associated with WRF in crude logistic regression models, and remained significantly associated when further adjusted for diabetes, SBP, ACEi, ARB, beta-blockers, prior HF, creatinine, and NT-proBNP/BNP with an OR 1.74 (1.20-2.53); p-value 0.004 in the pooled cohort.

Clinical outcomes

Results from bio-ADM and penKid's association with uni-variable logistic regression for in-hospital mortality, multi-variable linear regression for length of stay and multi-variable Cox regression analysis for re-hospitalization and one-year mortality can be found in **Table 10**.

Table 10. Association of bio-ADM and penKid with clinical outcomes

		Bio-ADM			PenKid	
	OR/β/HR	95% CI	p-value	OR/β/HR	95% CI	p-value
Pooled cohort						
In-hospital mortality (n=24)	OR 1.50	1.00-2.26	0.051	2.24	1.57-3.20	<0.001
Length of stay	β 0.702	-	<0.005	β 0.006	-	0.30
HARVEST-Malmö						
Re-hospitalization (n=188)	HR 1.15	1.06-1.47	0.007	1.10	0.93-1.30	0.27
1-year mortality (n=50)	HR 1.35	1.01-1.79	0.043	HR 1.35	1.04-1.77	0.027

Uni-variable logistic regression was performed for in-hospital mortality
For length of stay linear regression adjusted for age, sex, DM, systolic BP, atrial fibrillation, smoking and prior HF
For re-hospitalization and 1-year mortality Cox regression analysis adjusted for age, sex, DM, systolic BP, atrial fibrillation, smoking, NT-proBNP/BNP and prior HF
OR odds ratio; HR hazard ratio; β standardized beta-coefficient

Discussion

This discussion will attempt to present some unifying themes or mechanisms in CMD using the biomarkers studied in this thesis. However, the first part of the Discussion will be based mainly on the results from **Paper I** and **Paper II,** and the second part will be based predominantly on the results from **Paper III** and **Paper IV** (focused on biomarkers in the acute heart failure setting).

Cardiometabolic biomarkers

In **Paper I** we studied how 92 proteins were associated with incident DM. We identified 7 proteins with independent associations with incident DM, three of which, to our knowledge, were not previously reported. DM is often simplified to a combination of insulin resistance (IR) and insulin deficiency. However, this rather simplistic view of DM development was expanded by Defronzo to eight distinct mechanisms constituting an *ominous octagon* (86). This model was then further expanded with the addition of systemic inflammation(87) and impaired insulin mediated vasodilatation(88) to ten distinct pathophysiological abnormalities resulting in hyperglycemia and ultimately DM as illustrated in **Figure 5** (89) constituting not an ominous octet but a *destructive decagon*.

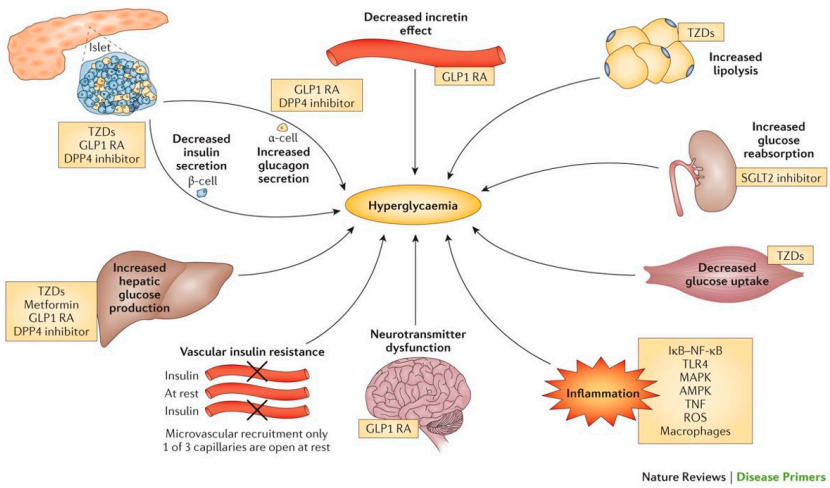

Figure 5. Pathophysiological mechanisms of hypergycemia (89) . With permission from SpringerNature.

Recent studies have also shown that the composition of the gut microbiota plays an essential and causal role in the development of DM (90, 91).

Genetic components

In addition to the abovementioned mechanisms there is also a strong genetic component in the development of DM. With the exception of a few rare variants, DM is a complex polygenic disease and genome wide association studies have identified more than a hundred common genetic variants associated with DM (92, 93). However, these variants only have modest effects and more than half of non-diabetic subjects also carry these risk variants (92, 94). Nonetheless, the identification of these risk variants, albeit their high prevalence and modest risk increase, has aided in the understanding of the pathogenesis of DM. The combination of risk variants into genetic risk scores showed that high genetic risk subjects were more likely to develop DM independently of traditional clinical risk factors (95).

Many of the mechanisms involved in development of DM are also applicable in CMD with multi-organ IR playing the central and driving role in CMD (96, 97). The following segments will highlight a few of these mechanisms using the biomarkers studied in this thesis as a vantage point.

Insulin resistance

IR lies at the core of DM and CMD. The skeletal muscle and liver are responsible for most of the glucose uptake after a meal and are both progressively resistant to the actions of insulin in CMD (97). In skeletal muscle, defects in insulin signalling, glucose transport and mitochondrial dysfunction all contribute to IR (89).

The homeostasis model assessment of insulin resistance (HOMA-IR) uses FPG and insulin concentration to assess IR with higher values indicating more severe IR (98) and is strongly linked to CVD (99). Conversely, high levels of *insulin-like growth factor binding protein-2* (IGFBP-2) have been shown to promote glucose uptake thereby reducing IR and are inversely associated with incident DM, as shown in **Paper I** and others before us (62, 100). Furthermore, in **Paper I** we showed an inverse association with IGFBP-2 and prevalent DM which has also been shown previously (101).

In animal studies, transgenic mice overexpressing human IGFBP-2 were resistant to the development of obesity and IR when fed a high-fat/high energy diet. Furthermore, they had decreased leptin levels, increased glucose sensitivity, and lower blood pressure compared to wild-type mice. The authors suggested a direct negative effect of IGFBP-2 on adipogenesis, thus perhaps playing a role in obesity prevention (102).

However, in **Paper II**, IGFBP-2 was associated with both ACM and CVM in adjusted models and with incident CE in age and sex-adjusted analyses (**Table 5**). Our findings are supported from a recently published study from the Framingham Heart Study (FHS), also using a proteomic approach, where IGFBP-2 was associated with both ACM and CVM as well as incident HF but not CE (103). These contradictive findings are rather perplexing as IGFBP-2 has been shown to be inversely associated with arterial stiffness as measured by pulse wave velocity, in itself a risk marker for CVD (101), and lower levels of IGFBP-2 have been associated with increased risk of the metabolic syndrome (104). The seemingly opposing role of IGFBP-2 in CMD and mortality warrants further studies.

Dyslipidemia and obesity

Obesity in an attempt to be less stigmatizing and more scientific, is sometimes referred to as adiposity-based chronic disease (ABCD), which is a complex process involving abnormalities in the amount, distribution and function of adipose tissue (105). IR is present not only in skeletal muscle and liver but also in the fat cells or adipocytes causing increased lipolysis. This leads to elevated FFA levels which in the liver not only stimulate gluconeogenesis leading to hyperglycemia, but also increases TG levels and clearance of anti-oxidative HDL(97). This further perpetuates IR in muscle and liver and is referred to as lipotoxicity (106).

The fat cells aren't passive containers of fat but produce cytokines or adipokines such as *fatty acid binding protein-4* (FABP4) that serve as a carrier of FFAs. In **Paper I** FABP4 was associated with incident DM as shown previously (107).

Genetic knockout mice without expression of FABP4 were developmentally normal and when fed a high-fat/high-energy diet developed obesity but not IR or DM unlike the control mice indicating that FABP4 is a central link between obesity and IR (108).

Elevated levels of FABP4 has further been associated with CVD in a population study (109) and increased CVM and CE in patients with prevalent CVD (110). This is in line with our findings from **Paper II** where FABP4 was associated with ACM, CVM and HF in age- and sex adjusted analyses and incident CE in the adjusted model.

Treatment with a FABP4-inhibitor in mice resulted in reduced atherosclerotic lesions and improved glucose control (111) and thus the development of specific monoclonal antibodies targeting FABP4 could also be an interesting therapeutic alternative (112).

Another central characteristic of CMD is dyslipidemia with high TG levels and low levels of HDL, both traditional strong risk factors for CVD (113). While HDL levels provide information about the amount of HDL, they don't necessarily reflect the

function or composition of HDL which is illustrated by the fact that half of patients who suffer a CE have normal or even elevated levels of HDL (114, 115).

In **Paper I** we found that higher levels of the HDL-bound protein *Paraoxonase-3* (PON-3) had a protective effect against incident DM in fully adjusted models including FPG suggesting a glucose-independent pathway. This inverse association of PON-3 with DM has later been confirmed in another Swedish study (116). Higher levels of PON-3 were also inversely associated with prevalent DM which is consistent with earlier findings that increased duration of DM is associated with reduced paraoxonase activity (117).

In **paper II** we saw that elevated levels of PON-3 were associated with reduced ACM and HF in age- and sex adjusted analyses which is consistent with two previous studies that showed that higher levels of PON-3 and paraoxonase activity was associated with reduced atherosclerotic burden measured by coronary angiography in diabetic subjects (117, 118). In vitro studies have shown that PON-3 not only prevents the formation of oxidized low-density lipoprotein cholesterol (LDL) but also inhibits its pro-inflammatory activity (119). Oxidized LDL is a marker for oxidative stress and has been associated with incident DM (120) thus, in some sense, closing the loop for PON-3 and its possibly unifying role in CMD.

Inflammation

Low-grade inflammation has been shown to precede and predict development of DM and CVD supporting the concept that chronic inflammation is a predictor of CMD development (87) (121, 122).

In **Paper I,** we confirmed the association previously presented in a Danish population study (123) of the macrophage specific protein *scavenger receptor cysteine rich type 1 protein M130* (CD163) with incident DM. CD163 is highly expressed in adipose tissue and may present a link between inflammation, obesity and CMD (124). CD163 has also been associated with increased coronary atherosclerotic burden independently of traditional risk factors (125) which is in line with our findings from **Paper II** of CD163 being associated with incident CE in age- and sex adjusted analyses and with ACM and CVM in fully adjusted models.

Furthermore, the lysosomal endopeptidase *Cathepsin D* (CatD) can possibly contribute to the chronic adipose inflammation seen in CMD by causing adipocyte apoptosis which is associated with IR (126). In a Swedish proteomic study, CatD was associated with IR in two large community cohorts (127). This is consistent with our findings from **Paper I** where CatD was associated with incident and prevalent DM. However, a Mendelian randomization study from the same Swedish group was unable to show any significant causal relationship for CatD and DM (116) but, as the authors themselves state, their study might have been

underpowered to show a causal link for the proteins tested in Mendelian randomization analysis.

Nonetheless, CatD still presents an interesting link in CMD as it has also been associated with incident CE (128) and in a proteomic study of 82 proteins, CatD was the strongest predictor of dysglycemia in an acute coronary syndrome (ACS) setting (129). In **Paper II** CatD was, along with Gal-4, the only protein associated with all investigated outcomes (ACM, CVM, CE, and HF) in adjusted models.

It has been suggested that CatD participates in the apoptosis of foam cells, a determinant of plaque instability (130). Furthermore, in patients with diabetes, CatD has been shown to truncate ApoA1 (the main protein of HDL) to ApoA1Δ (1-38) which binds to LDL and increases its susceptibility to oxidation, possibly contributing to the increased risk of CVD in diabetes (131) and to DM itself (120) similar to the effects of low paraoxonase activity described earlier.

In the ACS setting, previous studies of CatD are somewhat conflicting with elevated CatD levels being associated with myocardial infarction(132) but in another study of ACS-subjects, lower levels were associated with increased mortality and more severe CAD(133). Finally in a small study of ACS-subjects, CatD was elevated at admission compared to controls but at follow-up lower levels of CatD were associated with new-onset HF and recurrent CE (134). These results are based on small studies and hard to interpret. Further, our findings of CatD's associations with mortality and cardiovascular outcomes in a general population might not be comparable with the findings in populations that were in acute distress.

Inflammation and DM are considered pro-thrombotic states(135) and the anti-fibrinolytic protease inhibitor *plasminogen activator inhibitor-1* (PAI-1) has been associated with incident DM by us in **Paper I** (62) and in a recent meta-analysis (136). PAI-1 was borderline associated with incident CVD in the Framingham cohort (137) but we were unable to show any significant associations for PAI-1 regarding CVD or mortality in **Paper II.**

Incretin system

Oral glucose ingestion results in a larger insulin response than intravenous infusion of glucose which is called *the incretin effect* and is caused by the release, from the small intestine, of our two major incretin hormones; gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) (138). This incretin effect is significantly blunted or even absent in subjects with DM (139). Both GLP-1 and GIP stimulate insulin secretion but GLP-1 has several other cardiometabolic beneficial effects including suppression of glucagon, increased satiety, reduced blood pressure, improved dyslipidemia and an overall cardioprotective effect in CAD and HF (138, 140).

Galectin-4 (Gal-4) is a small protein in the gastrointestinal tract where it transports other proteins from the Golgi apparatus to the apical cell membrane of the enterocyte in a process called apical trafficking (141). One protein that is dependent on Gal-4 for its transportation is the protease dipeptidyl peptidase-4 (DPP-4)(141). In Gal-4-depleted mice, DPP-4 is misguided when transported and accumulates intracellularly, as opposed to being expressed at the apical membrane of the enterocyte in the presence of Gal-4 (141).

DPP-4 cleaves and inactivates GIP and GLP-1, which leads to several cardiometabolically adverse effects, including endothelial dysfunction, IR and dyslipidemia (142). Thus, the introduction of incretin-based antidiabetic medication in the form of DPP-4 inhibitors or GLP-1 agonists represented a major advance in DM treatment, without risks of hypoglycemia or weight gain (138). In the LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) trial the GLP-1 agonist liraglutide showed lower risk of CE and CVM and was the first anti-diabetic medication to be approved as therapy to reduce CVD in patients with DM (30). Liraglutide was followed by semaglutide, another GLP-1 agonist, which has also shown cardiovascular benefits (143). It should be noted however, that the use of DPP-4 inhibitors have not shown any cardiovascular benefits and has in fact elicited some concern due to increased hospitalization for HF and a recent meta-analysis consisting of both randomized clinical trials and observational studies suggested an increased risk in certain patients (144).

Nonetheless, as a novel finding, we have shown in **Paper I** that increased levels of Gal-4 are associated with prevalent and incident DM in fully adjusted models including FPG. In **Paper II,** Gal-4 was, as a novel finding to the best of our knowledge, associated with all investigated outcomes (CE, HF, CVM and ACM); all in fully adjusted models including NT-proBNP for incident HF.

As alluded to earlier, one possible explanation for our findings is that increased levels of Gal-4 lead to an increased expression of DPP-4 and thus reduced activity of the beneficial incretin GLP-1 as described above. As a last note, another Swedish research group from Sweden, also using the OLINK Proseek CVD III panel, found that Gal-4 was associated with aortic stenosis requiring surgery in two separate cohorts, and that Gal-4 in the discovery cohort was associated with aortic stenosis in the absence of CAD. Their findings could possibly be explained by the same mechanism described above, with increased levels of Gal-4 leading to increased activity of DPP-4, as DPP-4 has been shown to induce aortic valve calcification (145).

This concludes the first part of the discussion that was based on findings from **Paper I** and **Paper II.** The following part will try to maintain the cardiometabolic perspective but will be slightly more geared towards acute HF and results from **Paper III** and **Paper IV.**

Hypertension and endothelial dysfunction

There is a well-established relationship between IR and hypertension through several different mechanisms (146). The elevated levels of FFAs seen in IR and DM have a vasoconstrictive effect (147) and renal sodium absorption is increased in CMD (148).

Copeptin, which is the stable C-terminal fragment of the biologically active hormone *arginine vasopressin* is released from the pituitary gland in conditions of low plasma volume and low blood pressure. Elevated levels have been associated with hypertension, DM, CAD and mortality (71, 149-151). Based on these findings it has been suggested that disturbances in the vasopressin system measured through copeptin is a unifying possibly causal link in CMD (152).

Copeptin is highly predictive of adverse events in the acute HF setting (153). In **Paper III** we found that higher levels of copeptin was associated with post-discharge mortality in a fully adjusted model. This finding is supported by a recent meta-analysis consisting of 10 prospective cohort studies demonstrating that the predictive value of copeptin is comparable to NT-proBNP for all-cause mortality in HF patients (154). However, we could not identify a significant association with re-hospitalization that has been demonstrated earlier (153).

Based on the above described findings of copeptin's role in DM, a pilot study showed that increased water intake could reduce copeptin levels in healthy subjects (155) which propelled an ongoing clinical trial showing that water supplementation could reduce both copeptin levels and FPG thus possibly reducing the risk of DM (156).

Although copeptin was shown to be the best predictor of 90 day-mortality and re-hospitalization in a prospective multi-center study testing multiple biomarkers(157), the safety and efficacy of lowering copeptin by water supplementation in a state of fluid overload such as HF remains to be seen.

*Adrenomedullin (*ADM) is secreted from endothelial cells as a response to vascular wall stress and promotes vasodilation by increasing nitric oxide and decreasing endothelin-1 (158). It has also been shown to be a key determinant in regulating vascular integrity and endothelial permeability(159, 160) The elevated levels of ADM seen in hypertension are thought to be compensatory and protective as it promotes natriuresis and vasodilation (161). However, ADM also plays a part in glucose metabolism; when subjects are injected with ADM, it inhibits insulin release and thus causes hyperglycemia (162). Furthermore, elevated levels of ADM are seen in subjects with diabetes, possibly through hyperglycemia-induced altered vascular function (163). Midregional pro-adrenomedullin (MR-proADM) is secreted in equimolar amounts to ADM and is more stable and therefor preferably measured (78).

In **Paper III** we showed that MR-proADM was associated with increased post-discharge mortality in HF patients and in **paper IV** we showed that bio-ADM was associated with length of hospital stay and re-hospitalization and borderline associated with in-hospital mortality (p=0.051). MR-proADM has previously been shown to be superior to NT-proBNP in predicting post-discharge mortality in HF patients (164).

For bio-ADM, our findings are supported by an earlier study showing that higher levels of bio-ADM in HF patients were associated with a composite primary outcome consisting of death, re-hospitalization, emergency dialysis, cardiac arrest, respiratory failure, prolonged hospitalisation and ACS in 30 days.(165)

Furthermore in **Paper III** we found that MR-proADM was associated with increased right ventricular size suggesting increased pressure in the pulmonary circulation, and rat studies show that infusion of ADM is associated with decreased right ventricular and atrial pressures (166).

In **Paper IV** we found that bio-ADM was strongly associated with peripheral edema as a sign of congestion in HF patients. With the risk of comparing apples to oranges, it is possible that the increased levels of MR-proADM is a compensatory mechanism to decrease pulmonary pressure and counteract the right ventricular dilatation but instead resulting in vasodilation which is manifested as peripheral edema, as seen in **Paper IV.**

Presently there are no biomarkers to assess congestion adequately. Congestion is the dominating reason patients are admitted to the hospital for HF and residual congestion at discharge is associated with high mortality and re-hospitalization (167). Natriuretic peptides can be unreliable in assessing congestion (168, 169) which is consistent with findings (unpublished) from **Paper IV** where NT-proBNP/BNP was not associated with any sign of congestion (peripheral edema, rales, CXR findings and dyspnea) in age- and sex adjusted analyses.

Currently, to assess congestion, the ESC recommends daily clinical evaluation of signs of congestion (170), which is inexact and subject to inter-observer variability. Thus, there is a need for a reliable and objective method to monitor the presence of congestion.

A Dutch study showed a significant decrease in bio-ADM in HF patients with little or no residual congestion after 1 week, compared with patients with significant residual congestion. Congestion was assessed by a comprehensive graded composite score of clinical and laboratory values(171). Several studies followed in supporting bio-ADM as a potential novel biomarker of congestion and predictor of adverse clinical events that can possibly aid in the guidance of treatment (172, 173).

Endothelin-1 (ET-1) like ADM, is secreted from the endothelial cells but contrary to ADM, ET-1 is a potent vasoconstrictor and has been associated with CVD including hypertension, HF and CAD (174). Furthermore, ET-1 has been implicated

in promoting IR and diabetes-related vascular complications by inhibiting glucose uptake in skeletal muscle cells and adipocytes, stimulating lipolysis and the release of pro-inflammatory cytokines (175). Moreover, unpublished data from the ESC Digital Congress 2020 showed that increased levels of ET-1 are associated with incident DM independently of FPG, suggesting an glucose-independent pathophysiological pathway (176).

C-terminal proendothelin-1 (CT-proET-1) is an inactive cleavage fragment from ET-1 and also more stable and therefor preferably measured (79). In **Paper III** we showed that CT-proET-1 was borderline associated with post-discharge mortality in age- and sex adjusted analyses which is supported by a meta-analysis comprising of 32 studies and almost twenty thousand subjects that showed increased levels of CT-proET-1 were associated with worse prognosis and increased mortality in HF patients (177).

The role of ET-1 in pulmonary arterial hypertension (PAH) is well-established with increased levels leading to pulmonary vasoconstriction (178) which can help explain our findings from **Paper III** where CT-proET-1 was associated with right ventricular dilatation.

PAH is often treated with endothelin-receptor antagonists (ETRA) and studies have shown that such treatment also improves insulin sensitivity (179) and based on ET-1's role in CMD, it has been suggested that it may be beneficial to widen the treatment indication for ETRAs beyond PAH(180).

Renal aspects in cardiometabolic disease

Chronic kidney disease is a strong risk factor for CVD (181) and diabetic nephropathy is the leading cause of end-stage renal disease (182). In IR and DM, the kidneys' ability to reabsorb glucose from the urine is augmented in a maladaptive effort to conserve glucose. This mechanism is of particular interest since the advent of inhibitors of the sodium glucose co-transporter 2 (SGLT-2) that not only have a glucose lowering effect but also very promising cardio- and renoprotective effects (183-186).

Cystatin C is a well-established marker for renal function superior to creatinine (187) and a well-known predictor of CVD (188). Furthermore, cystatin C levels are significantly higher in subjects with DM (189) and has been linked to incident DM and CMD (190), but a causal association between cystatin C and DM or CVD has been challenged through negative Mendelian randomization studies (191, 192). In **Paper III** cystatin C was the strongest predictor of mortality highlighting the massive impact renal dysfunction has on prognosis in HF.

The prognostic significance of renal function was also shown in **Paper IV** where penKid was associated with worsening renal function (WRF), in-hospital mortality

and 1-year mortality and its prognostic utility for CVM and hospitalization has previously been shown (193).

PenKid is a stable breakdown product of endogenous opiods (i.e. enkephalins) and has emerged as a highly dynamic marker of acute kidney injury in HF and sepsis (70, 194). PenKid is considered an inflammation-independent marker of kidney function that allows the early diagnosis of acute kidney injury by predicting the future change in serum creatinine (195).

One possible mechanism for penKid, both in WRF and its prognostic value in mortality, might be related to the cardiodepressive effects of enkephalins(196). This theory is indirectly supported by the fact that administration of opiates to HF patients resulted in worse prognosis in HF patients even after adjustment for clinical presentation and vital parameters (197). From a CMD perspective, based on experimental studies there has been speculation that an increased sensitivity to enkephalins might be important in the pathogenesis of DM (198) but no studies pertaining to pro-enkephalin's potential part have been found.

Natriuretic peptides

Brain natriuretic peptide (BNP) or its more stable fragment NT-proBNP is released from the cardiac ventricles in response to mechanical and neurohormonal stimuli and is central in the diagnosis and prognosis of HF (170). From a cardiological perspective NPs have a number of beneficial effects including reduced myocardial hypertrophy, arterial vasodilation, increased natriuresis and reduced activity of the renin–angiotensin–aldosterone system (199). The elevated levels of NPs that are associated with worse prognosis in HF and in CAD independent of concomitant HF thus seem paradoxical but are thought to be compensatory and the result of a relative deficiency and resistance to BNP (200), not unlike the high levels of insulin seen in DM and IR.

We were unable to find a significant association for NT-proBNP and incident DM in **Paper I** but in **Paper III,** however, NT-proBNP, as expected, was associated with post-discharge mortality and was the only biomarker that independently associated with re-hospitalization.

There is an inverse association between NPs, obesity and DM (201) possibly through increased clearance and degradation by the peptidase *neprisylin* which is expressed at increased levels in obesity (202, 203). In a post-hoc analysis of the *Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and morbidity in Heart Failure* (PARADIGM-HF) trial (204) treatment with sacubitril/valsartan, a combined angiotensin receptor-neprilysin inhibitor, resulted in lower long-term HbA1c reduction suggesting a role for neprilysin inhibition in glycemic control (205).

Furthermore, Mendelian randomization studies provide evidence for a potential causal role of BNP in DM with lower levels increasing risk of DM (206). In addition, low levels of BNP's cousin *atrial natriuretic peptide* which is released from the atria in the heart has been associated with incident DM and confirmed in Mendelian randomization studies suggesting a causal association (207, 208).

Future perspectives

CMD is the leading cause of mortality and morbidity worldwide and poses an enormous challenge to health care systems. While Kannel's seminal work on identifying risk factors for CVD from the Framingham cohort (209) has provided a framework; traditional risk factors while potent (210), are imperfect in their predictive ability. This is illustrated by the fact that many patients presenting with an ACS lack any of the conventional risk factors (211) and in the case of DM it has been shown in both Swedish and Japanese populations that the cardiometabolic process begins more than 20 years before actual diagnosis is made (212, 213).

This disguised presence of CMD in the absence of conventional risk factors stresses the need for novel biomarkers that provide additional value to traditional risk factors and can provide new insights into the underlying pathophysiological mechanisms.

Wang et al performed an interesting simulation adding up to a 100 hypothetical biomarkers to a traditional risk factor model for CVD. They found that the most important element to improving the model's predictive ability was the correlation or rather lack thereof, of the hypothetical biomarkers. It took 50 moderately correlated biomarkers to improve the model but less than 10 if they were weakly correlated (214).

I believe this creates a rationale for exploratory studies, such as ours, of novel pathophysiological pathways in CMD that may someday have diagnostic, prognostic, and therapeutic implications.

In **Paper I** we identified Gal-4 and PON-3 as two novel associations for incident DM independently of glucose. Since most screening measures for DM and IR (FPG, OGTT and HbA1c) naturally are based on glucose, in future studies it would be intriguing to see how other biomarkers perform in identifying high-risk but normoglycemic individuals that may benefit from heightened clinical vigilance, lifestyle intervention or even pharmacological treatment. In the case of Gal-4 and the results from **Paper II** where it was associated with all investigated outcomes and given its possible role in the incretin system, if one were to speculate, it may be of value of initiating treatment with a GLP-1 agonist sooner rather than later.

In general, using a biomarker approach when choosing between treatment options addresses the pursuit of personalized medicine and will hopefully provide the precision desired to successfully treat our patients in the future.

For many clinicians, including myself, there are many times when it is frustrating how difficult it is to assess the congestion status of a patient admitted for HF. We auscultate the lungs for rales, check the legs for edema and, if still ambitious, look for presence of jugular venous distension. Sometimes we resort to more advanced methods such as chest X-ray, pulmonary ultrasound or measuring natriuretic peptides but still the question remains; is the patient "dry" or "wet"? If we are too aggressive with diuretics we risk jeopardizing renal function and if we're not aggressive enough we prolong the patient's suffering or even worse, given today's scarcity of hospital beds, the length of hospital stay. Obviously a biomarker is needed.

Bio-ADM has emerged as a biomarker for residual congestion and the clinical and prognostic consequences that entails. Thus, guiding decongestive treatment i.e. diuretics, with bio-ADM possibly in conjunction with a highly dynamic marker for renal function such as penKid, could be of assistance to the treating clinician and ultimately the patient.

As mentioned earlier, vasodilation and vascular integrity are the most important effects of adrenomedullin but it's believed that its effect depend on its location (215). Whilst intravascular ADM promotes vascular integrity and reduce endothelial permeability which would be beneficial in an acute HF setting; interstitial ADM has the opposite effect promoting vasodilation which is detrimental in situations of low-perfusion such as cardiogenic shock or sepsis (216, 217). As illustrated in **Figure 6,** a monoclonal antibody, adrecizumab, targeting bio-ADM and then trapping and translocating it intravascularly has been developed (218) constituting a novel treatment strategy and currently phase II studies in HF are being initiated.

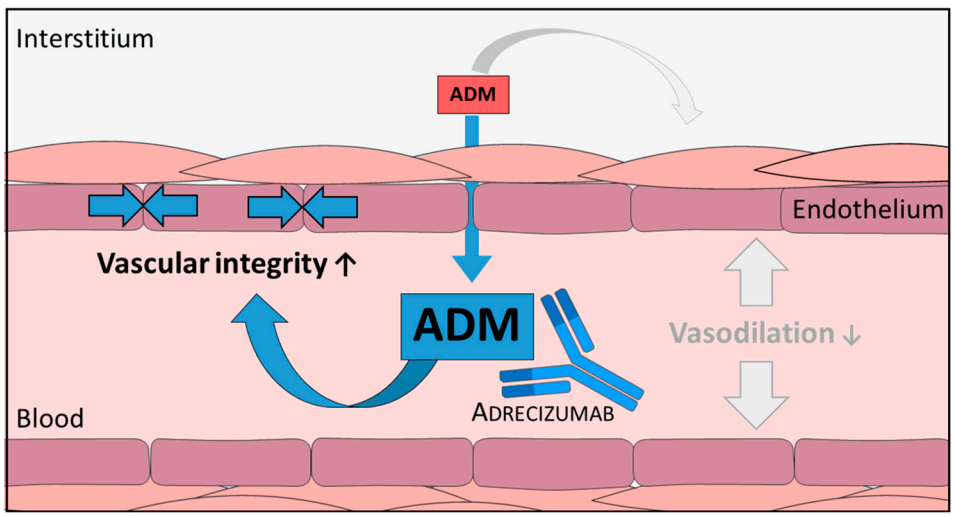

Figure 6. Role of adrenomedullin in the intra- and extravascular space and mode of action for adrecizumab. With permission from Adrenomed AG.

Popular summary in Swedish

Biomarkörer inom kardiometabol sjukdom

Det finns ett väldigt starkt och välkänt samband i befolkningen mellan diabetes och hjärtkärlsjukdom men de exakta underliggande mekanismerna är ännu inte helt klarlagda. I takt med en åldrande befolkning och en förändrad livsstil ser vi en dramatisk ökning av pandemiska proportioner av dessa sjukdomar. En del av hjärtkärlsjukdom är hjärtsvikt som innebär att hjärtat inte kan förse kroppens behov av blod och detta yttrar sig ofta i form av andfåddhet, trötthet och bensvullnad. De vanligaste orsakerna till hjärtsvikt är kranskärlssjukdom och högt blodtryck men även diabetes är en viktig och stor risk faktor. Cirka 3 procent av Sveriges befolkning lider av hjärtsvikt och det är den vanligaste orsaken till sjukhusvård. Trots stora behandlingsframsteg så är prognosen fortsatt dyster och dödligheten motsvarar många former av allvarlig cancer.

En biomarkör kan enklast definieras som något mätbart och objektivt och något som ger information om en patients hälsotillstånd. Detta i motsats till symptom som är patients subjektiva upplevelse av en sjukdom. Biomarkörer kan bland annat användas för att påvisa eller utesluta sjukdom, utvärdera sjukdomsutveckling eller behandlingseffekt.

Proteomik beskriver mönstret av proteiner hos en människa vid olika tillstånd och är ett spännande sätt att kartlägga vilka proteiner och i vilken mängd som uttrycks hos människor som till exempel utvecklar en viss sjukdom.

Det övergripande syftet med den här avhandlingen var att studera olika proteiner, vissa redan etablerade som biomarkörer och vissa mer experimentella, och deras effekt på insjuknande i diabetes och hjärtkärlsjukdom och i gränslandet däremellan som ibland benämns kardiometabol sjukdom.

I de två första artiklarna använde oss av Malmö Förebyggande Medicin som var ett primärpreventivt projekt som startade 1974. Under 17 år blev totalt 33000 män och kvinnor undersökta med blodprover, frågeformulär och kroppslig undersökning. Cirka 25 år senare erbjöds de kvarlevande deltagarna att delta i en liknande återundersökning där ca 18000 deltog. Från dessa valdes knappt 2000 deltagare slumpmässigt ut och genomgick ultraljudsundersökning av hjärtat och utvidgad blodprovstagning och det är denna grupp vi har använt oss av i våra studier.

Deltagarna har sedan dess följts i nationella register för diabetes, hjärtkärlsjukdom och död.

I det tredje och fjärde arbetet har vi använt oss av HARVEST-studien (HeARt and brain failure inVESTigation trial) som är en pågående studie av patienter inlagda för hjärtsvikt vid Kardiologiska och Internmedicinska kliniken vid Skånes Universitetssjukhus, Malmö. Patienterna får genomgå ett stort batteri av tester både på hjärtat men även på deras kognitiva förmågor. För närvarande är cirka 500 patienter inkluderade.

I det första arbetet använde vi oss av en särskild proteomisk analys som möjliggör att man kan analysera nästan 100 olika proteiner i en mikroliter plasma och vi kunde identifiera 7 proteiner som var förknippade med insjuknande i diabetes varav 4 av proteiner inte tidigare fanns beskrivna i litteraturen.

I det andra arbetet byggde vi vidare på resultaten från det första arbetet och undersökte hur dessa 7 proteiner var förknippade med hjärtinfarkt, hjärtsvikt och dödlighet och fann att två proteiner, Galectin-4 och Cathepsin-D, var förknippade med samtliga utfall vilket inte tidigare fanns beskrivet. Galectin-4 har tidigare kopplats samman med inkretiner som är hormoner som frisätts från magtarm-kanalen då den utsätts för socker och syntetiska inkretiner används idag som läkemedel mot diabetes men har även i vissa fall visat sig minska risken för hjärtkärlsjukdom. Cathepsin-D är ett litet protein som klyver och i viss mån inaktiverar de positiva antioxidativa effekterna av HDL som ibland brukar benämnas som det goda kolesterolet.

I det tredje arbetet övergick vi till hjärtsviktspatienter i HARVEST-studien och undersökte fem olika proteiner som är involverade i de olika kompensatoriska mekanismer som träder i kraft när kroppen drabbas av hjärtsvikt. Vi fann att förhöjda nivåer av fyra av proteinerna var förknippade med en ökad risk för död efter utskrivning men endast ett protein, NT-proBNP, var förknippat med ökad risk för återinläggning. NT-proBNP är idag den mest använda biomarkören för hjärtsvikt både som screening och i viss mån för att bedöma behandlingseffekt.

I det fjärde och avslutande arbetet undersökte vi två tämligen nya biomarkörer, bio-ADM och penKid. Bio-ADM frisätts från kärlväggen och har en stärkande effekt på kärlväggen när det befinner sig inuti kärlbanan men när det befinner sig utanför kärlväggen har det en avslappnande effekt på de muskelceller som omger kärlväggen vilket leder till ett ökat vätskeutträde och kan ge upphov till bland annat ökad bensvullnad vid hjärtsvikt. Bio-ADM har därför föreslagits som en ny biomarkör för kvarvarande övervätskning vid hjärtsvikt och kan hjälpa den behandlande läkaren om patienten är i fortsatt behov av vätskedrivande behandling. PenKid är en biomarkör för njurfunktion och signalerar väldigt tidigt om njurfunktionen börjar ta skada vid till exempelvis aggressiv behandling med vätskedrivande läkemedel. Kombinationen av dessa biomarkörer är därför särskilt tilltalande för den behandlande läkaren då man kan få stöd huruvida patienten är

fortsatt övervätskad, vilket kan vara en väldigt svår klinisk bedömning, och därmed i behov av ytterligare vätskedrivande terapi samtidigt som man får en tidig signal om njurfunktionen börjar påverkas. Vi kunde visa att förhöjda nivåer av bioADM var förknippat med övervätskning men även förlängd sjukhusvård och ökad risk för återinläggning medan penKid var förknippat med ökad dödlighet både under sjukhusvistelsen och efter utskrivning.

Errata

Paper III

In the Results section, subheading *Biomarkers and mortality,* we state that all five biomarkers were associated with post-discharge mortality in age- and sexadjusted Cox regression analyses. However, as seen in Table 2 the p-value for CT-pro-ET-1 is 0.014 and thus did not meet the pre-specified Bonferroni-corrected p-value of <0.01 (0.05/5).

Paper IV

In table 1 under *Peripheral oedema* the correct number for the pooled cohort should be n=360 (215+145).

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